B6

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 1/00

A1

(11) International Publication Number: WO 00/32619

(43) International Publication Date: 8 June 2000 (08.06.00)

(21) International Application Number: PCT/US99/27839

(22) International Filing Date: 24 November 1999 (24.11.99)

(30) Priority Data:
60/110,359 30 November 1998 (30.11.98) US

(71) Applicant: RIBOGENE, INC. [US/US]; 26118 Research Road, Hayward, CA 94545 (US).

(72) Inventors: DAMMEL, Carol, S.; 1902 Winding Creek Court, San Jose, CA 95148 (US). WATSON, Julie, C.; 1470 Santa Inez Drive, San Jose, CA 95125 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFICATION OF INHIBITORS OF RIBOSOME ASSEMBLY

(57) Abstract

The invention provides a means for identifying novel antibiotics by screening for compounds that inhibit bacterial ribosome assembly and ribosomal protein synthesis, which comprises the screening of test compounds to identify those that inhibit the activity of a bacterial ribosomal protein. The *in vitro* and *in vivo* assays detect (a) increased translation of a reporter mRNA that is normally repressed in the presence of a ribosomal protein, (b) increased growth in cells that over–express ribosomal protein, and (c) decreased binding of the ribosomal protein to its target RNA. The invention encompasses the (a) methods of screening and testing compounds by the above methods, (b) compounds that are identified in the assay which inhibit ribosome assembly and protein synthesis and (c) methods for treatment using said compounds.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS AND COMPOSITIONS FOR IDENTIFICATION OF INHIBITORS OF RIBOSOME ASSEMBLY

1. INTRODUCTION

The present invention relates to methods for identifying novel antibiotics by screening for compounds that inhibit ribosome assembly in bacteria. The invention relates to targeted, efficient, and high-throughput screens to identify small molecules, compounds, peptides, etc. that interfere with RNA-protein interactions required for bacterial ribosomal protein synthesis and ribosome assembly. Such screens encompass both *in vivo* and *in vitro* assays. The invention further encompasses antibiotic candidate compounds identified using such screening methods.

2. BACKGROUND OF THE INVENTION

New antibiotics are desperately needed. The widespread use of antibiotics over the past half century has lead to the emergence of bacterial strains that are resistant to nearly all antibiotics now in use. Thus there is an immediate need to develop fast and efficient methods for producing new antibiotics to combat the increasing number of these antibiotic-resistant strains (Chopra et al., 1997, Antimicrob. Agents Chemother., 37:1563-1571; Cohen, 1992, Science, 257:1050-1055; Kunin, 1993, Ann. Intern. Med., 118:557-561; Neu, 1992, Science, 257:1064-1073; Tenover & Hughes, 1996, JAMA, 275:300-304). Ideally, new classes of antibiotics can be discovered that will be toxic to a broad range of pathogenic bacteria, and, at the same time, be harmless to their human hosts. It is also hoped that these new antibiotics will target cell components and processes other than those targeted by existing antibiotics, so that resistant strains would not already be immune to the new drugs.

Traditional approaches to antibiotic development have failed to meet these needs. One commonly used approach involves chemical modification of an existing antibiotic to produce a more potent formulation. Another approach involves screening for compounds that target the resistance mechanism of a known antibiotic. Such compounds are then be used in conjunction with the known antibiotic to improve its efficacy. These

approaches have been somewhat successful, but are research intensive and such drugs tend to target the same bacterial processes as existing antibiotics, and thus, like the earlier breed of antibiotics, are likely to quickly encounter resistance. A second approach has involved mass screening of compounds for their ability to inhibit bacterial growth. Using microbiological assays, natural products and semisynthetic or synthetic chemicals are screened for their ability to kill or arrest the growth of a target pathogen. At least initially, this approach has the advantage of being simple and relatively inexpensive, and allowing rapid testing of large libraries of compounds. However, the promising lead compounds that emerge from such screens subsequently must be tested for host toxicity. Furthermore, since such screens are result-oriented and blind to mechanism, further studies must be done in order to precisely understand the drug's mechanism of action and to identify its target in the cell.

A powerful new approach, called "targeted screening," has recently become possible with the advent of the field of genomics and our increasing understanding of bacterial molecular genetics. Using this approach, one can select a molecule or cellular process that might be useful as a drug target, and design screens to identify compounds that interfere with such molecule or process. Like simple growth inhibition assays, targeted screening can be fast and inexpensive, and can allow rapid high-throughput screening of a large number of compounds. Moreover, it has the additional advantage that the drug's target is known in advance, reducing the necessity of subsequent drug development. The remaining challenge, and more serious one, is finding the right molecule to target to design antibiotics that will impact a broad spectrum of pathogens, while remaining harmless to the cells of the host organism.

The genomes of several pathogenic microorganisms, such as *Escherichia coli*, *Helicobacter pylori*, and *Chlamydia trachomatis*, recently have been sequenced (Blattner et al., 1997, Science 277: 1453; Tomb et al., 1997, Nature, 388: 539-547). The availability of gene sequences encoding all proteins of these bacteria provides an unprecedented opportunity for understanding and manipulating bacterial genomes at the molecular level. A number of genes are known or are suspected to be essential to growth, survival or virulence. Such genes could be ideal targets in screening for novel antibiotics.

Antibiotics that act against the protein biosynthetic machinery, *e.g.*, tetracyclines, chloramphenicol, erythromycin and streptomycin, are valuable in treating bacterial infections (Gale et al., 1981, The molecular basis of antibiotic action 2nd ed., John Wiley & Sons, London, United Kingdom; Russell & Chopra, 1996, Understanding antibacterial action and resistance, 2nd ed., Ellis Horwood, New York, NY). For example, erythromycin and other macrolide antibiotics are effective against both gram positive and gram negative bacteria. However, erythromycin-resistant strains of *Streptococcus* and *Staphylococcus* are known, and may be on the rise. The exact mechanism of action of macrolide antibiotics still is not completely understood, although it has been suggested that erythromycin inhibits a step in translation between initiation and elongation (1984, Omura S., in Macrolide Antibiotics, Orlando, FL, Academic Press).

One important step in protein biosynthesis is the translation of genetic information from messenger RNA (mRNA) to proteins, an essential cellular process for all living organisms, including infectious pathogens. Translation requires the coordinated interplay of mRNA, ribosomes, tRNAs, and a number of specialized proteins (Hill et al., 1990, The Ribosome. Structure, Function and Evolution. Washington, DC: Am. Soc. Microbiol.; Nierhaus et al., 1992, The Translational Apparatus. Structure, Function, Regulation, Evolution. New York: Plenum.; Matheson et al., 1995, Biochem. Cell Biol. 73:739-1227; Zimmermann et al., 1996, Ribosomal RNA. Structure, Evolution, Processing, and Function in Protein Biosynthesis. Boca Raton, FL: CRC).

The *E. coli* ribosome, which has been the subject of intense research for over 30 years, is composed of two large particles, the 30S and the 50S subunits. The 30S subunit consists of a 16S rRNA molecule and 21 small ribosomal proteins ("r-proteins'). The 50S subunit is composed of two ribosomal RNA (rRNA) molecules, 23S and 5S rRNA, and 31 large ribosomal proteins.

While prokaryotic ribosomes are similar, regardless of species, they differ in many respects from eukaryotic ribosomes. In general, mammalian ribosomes are more complex than their bacterial counterparts. Mammalian rRNAs are larger – 18S and 28S as compared to 16S and 23S for bacterial rRNAs. There are many fewer r-proteins found in *E. coli* ribosomes (52) than in eukaryotic ribosomes (70-82). Similarly, ribosomal proteins from different species of bacteria are closely related, but show little to no similarity to

ribosomal proteins from higher and lower eukaryotes. Eukaryotic mitochondria also encode r-proteins, but these r-proteins show no significant relatedness to bacterial proteins (Wittmann-Liebold et al., 1990, in W.E. Hill et al., eds., The Ribosome: Structure, Function, and Evolution. A.S.M. Press Washington D.C.).

Ribosomal RNAs, on the other hand, are one of the most highly conserved classes of macromolecules known. rRNAs are few in number and yet play an important role in protein synthesis (Gutell et al., 1985, Prog. Nucleic Acid Res. Mol. Biol. 32:155-216).

Ribosome assembly in bacteria is a tightly controlled process. For example, the synthesis of ribosomal components, rRNA and r-proteins, is coordinately regulated to ensure that these molecules are produced in the optimal stoichiometry. Protein-RNA interactions play important regulatory roles at several steps in this process. Synthesis of r-proteins is negatively regulated at the translational level by the binding of repressor r-proteins to specific sites in mRNA. As part of another regulatory step in the ribosome assembly process, certain r-proteins bind to rRNA, to initiate the ordered assembly of the ribosome. Binding of these r-proteins, termed "primary binders," is required for the subsequent steps of ribosome assembly (Zengel & Lindahl, 1994, Prog. Nuc. Acid Res. Molec. Biol. 47:332-370).

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for identifying compounds with antibiotic activity. The invention is based, in part, on the discovery by the inventors that the process of ribosome assembly is an attractive target for drug discovery. The methods of the present invention are designed to identify inhibitors of ribosome assembly, and compounds that interfere with regulation of ribosomal protein synthesis.

Specifically, the invention relates to methods and compositions for sensitive and high-throughput screening assays to identify compounds that block the ribosomal protein-RNA interactions required for ribosome assembly and ribosomal protein synthesis. The invention encompasses both *in vivo* and *in vitro* assays to screen small molecules, compounds, recombinant proteins, peptides, nucleic acids, antibodies, etc. which interfere with ribosomal protein – RNA interactions.

The invention provides *in vivo* assays to identify test compounds that interfere with the specific binding of an r-protein to an RNA binding site. The invention encompasses a method for screening for a test compound wherein the translation of a reporter mRNA in a test cell contacted with a test compound is compared to the translation of the reporter mRNA in a test cell not contacted with the test compound. An increase in translation in the cell contacted with a test compound indicates that the test compound inhibits the activity of the respective r-protein in the test cell. In one specific embodiment of this assay, a reporter gene in the test cell produces the reporter mRNA.

The invention further encompasses an *in vivo* assay to screen a test compound based on measuring the growth of a test cell comprising an excess of a specific ribosomal protein. An increase in growth of a test cell contacted with the test compound, relative to a test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein.

In specific embodiments, the *in vivo* assays utilize an *E. coli* test cell. In another specific embodiment, the ribosomal protein is S8. In another specific embodiment of the method, the ribosomal protein can be S1, S4, S7, S15, S20, L1, L4, L10, or L20. In yet another embodiment, the reporter gene used in the *in vivo* assays is luciferase, chloramphenicol acetyl transferase, green fluorescent protein, β-galactosidase, β-lactamase, or β-glucuronidase.

The invention also provides compositions to be used in the screening assays of the invention. The invention provides a test cell comprising the reporter gene construct and all the factors necessary for transcription and translation of the reporter gene.

The invention further provides *in vitro* assays. In an *in vitro* reaction, the reporter mRNA is incubated with an r-protein, a test compound and the factors necessary for translation of the reporter mRNA. A test compound is contacted with a reaction mixture comprising a bacterial repressor ribosomal protein and a reporter messenger RNA. The translation of a reporter mRNA is measured. An increase in the translation of the reporter mRNA in the presence of the test compound indicates that the test compound inhibits the activity of the repressor ribosomal protein.

In a specific embodiment, the *in vitro* reaction mixture comprises an excess of a recombinant repressor ribosomal protein. In another specific embodiment, a signal produced by a reporter gene is used to measure the product of translation of the test mRNA.

The invention further provides *in vitro* physical binding assays which include filter binding assays, affinity capture binding assays, and chemical probing assays. The *in vitro* physical binding assays include methods for screening test compounds by measuring the formation of a complex between a bacterial primary binding ribosomal protein and an RNA molecule comprising an RNA binding site for the ribosomal protein. A decrease in the binding of the ribosomal protein to the RNA molecule contacted with the test compound, relative to the binding of the ribosomal protein to the RNA molecule not contacted with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA binding site.

In one specific embodiment, the ribosomal protein used in the *in vitro* binding assays is S8. In another specific embodiments, the ribosomal protein used in the *in vitro* binding assays is S4, S7, S15, S17, S20, L1, L2, L3, L4, L7/12, L10, L11, L15, L20, L23, or L24. In yet another specific embodiment, where an affinity capture binding assay is used, the ribosomal protein comprises an affinity tag. In yet another embodiment, a variety of chemical and enzymatic assays are used to probe the precise alteration in the RNA-protein interaction caused by introduction of the test compound.

The invention further encompasses methods for inhibiting the growth of bacteria by contacting the bacteria with a compound that inhibits the activity of the ribosomal protein identified using the *in vivo* and *in vitro* assays of the invention.

The invention further relates to pharmaceutical compositions comprising novel compounds that inhibit the activity of the repressor ribosomal protein, said compounds identified using the *in vivo* and *in vitro* assays of the invention.

The invention further encompasses methods for treating a patient with an infectious disease by administering to the patient a therapeutically effective amount of a compound that inhibits the activity of the ribosomal protein identified using the *in vivo* and *in vitro* assays provided herein.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. The organization of ten ribosomal protein operons in *E. coli*. The repressor r-protein for each operon is underlined. The positions of their mRNA binding sites are indicated by double asterisks.

FIG. 2. The alignment of S8 amino acid sequences from a number of prokaryotic organisms.

FIG. 3. The sequence and secondary structure of S8 RNA binding sites in *E. coli* and *H. influenzae*. The similarity of the base pairing within the double-stranded regions of the conserved mRNA and rRNA stem-loop-stem secondary structures is shown.

FIG. 4. The sequence and secondary structure of the *E. coli* S8 RNA binding site and a hybrid S8 binding site analog (arrow indicate start condons). A. The stem secondary structure of the S8 mRNA binding site. B. The S8 16S rRNA binding site. C. A hybrid S8 RNA binding site analog. The S8 hybrid RNA binding site shown here is used in the example presented in Section 6.

FIG. 5. The native S8 mRNA regulatory region located within the *spc* operon. A. Schematic diagram of the primary sequence of the S8 mRNA regulatory region. The stop codon of L24, the Shine Delgarno sequence, and start codon of L5 are indicated in bold type. B. Sequence of the S8 binding site on the mRNA. S8 binds to these regions (underlined in part A) thereby preventing translation of the *spc* operon.

FIG. 6. The S8 mRNA reporter construct. A. Schematic representation of the construct used in the *in vivo* and *in vitro* translation assays. The construct includes a strong Shine-Delgarno sequence (SD), a mini open reading frame (orf), a second SD, the hybrid RNA binding site with initiator AUG, and the Renilla Luciferase reporter gene. B. The nucleotide sequence of the RNA binding region. The underlined sequence represents the sequence of the S8 RNA binding site. Details are given in the example presented in Section 6.

FIG. 7. *In vitro* translation assay. The amount of S8 protein added to *in vitro* translation assays is indicated along horizontal axis. Renilla luciferase units (RLU) are indicated on vertical axis. Details are found in the example presented in Section 6.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for screening compounds with antibiotic activity. Specifically, the invention relates to assays for identifying compounds that interfere with assembly of bacterial ribosomes, either by blocking the assembly process, or by disrupting the regulated synthesis of ribosomal proteins (r-proteins). The invention is based on the discovery by the inventors that the process of ribosome assembly is an attractive target in searching for antibiotic candidates. The assays have been carefully designed to target a process that, while essential in bacteria, is sufficiently different from mammalian systems to yield antibiotics that are effective at arresting bacterial growth but safe for use in higher organisms. Moreover, because ribosome assembly is distinct from known antibiotic targets, the antibiotics that are identified through the methods of the present invention are likely to be structurally novel, without pre-existing resistances. And, in contrast to simple growth inhibition screening, the assays target specific processes and components of ribosomal assembly, thus obviating the need for subsequent mechanism of action studies.

The interaction of ribosomal proteins with RNA is a central theme that influences the synthesis of ribosomal proteins and their assembly into fully functional ribosomes. Ribosomal assembly in *E. coli* requires the coordinate expression of rRNA and r-proteins. Thus, the proper regulation of r-protein synthesis is critical to ribosomal function. One regulatory mechanism that *E. coli* employs involves the negative feedback regulation of r-protein gene expression by the r-proteins themselves. The r-protein genes of *E. coli* are organized into groups of genes, called operons, that produce polycistronic mRNAs, often coding for a number of r-proteins. After its synthesis, a repressor r-protein binds to its own mRNA, thereby blocking translation. As a result, the translation of the whole set of r-proteins encoded by a single mRNA transcript is regulated by a single r-protein, termed the repressor r-protein. The repressor r-proteins for ten *E. coli* ribosomal protein gene operons as well as the locations of their mRNA binding sites are shown in Figure 1.

Binding of certain ribosomal proteins to ribosomal RNAs (rRNAs) is also necessary for the ordered assembly of fully functional ribosomes. In the course of

assembly, a subset of ribosomal proteins, termed primary binding r-proteins, bind rRNA directly, and facilitate the binding of other ribosomal proteins.

Because the interaction of r-proteins with RNA is critical for proper ribosomc function, and because proper ribosomal function is essential for the growth of bacterial pathogens, thc inventors rccognize that compounds which can interfcre with the binding of ribosomal proteins to RNA are attractivc candidates for antimicrobial drugs. In light of the high degree of conservation among ribosome components within the bacteria, targeting ribosome assembly will yield antibiotics with activity against a broad range of bacterial species. Moreover, such drugs will be highly specific against bacteria with little or no effcct on mammalian ribosomes since it appears that there is little structural homology bctween the ribosome components of bacterial and eukaryotic organisms. Therefore, the interactions between ribosomal proteins (r-proteins) and their RNA binding sites are used as targets in the screening assays of the present invention.

RNA– protein interactions are important in a variety of cellular processes and are the subject of intense study. RNA molecules form complex secondary structures in solution (sce, for example, Doudna, 1997, 388:830-1; Ramos et al., Curr. Opin. Struct. Biol., 1997, 7:317-23). In contrast to DNA, where protein–DNA recognition can often be predicted based on the primary sequence of the DNA molecule, the secondary structure of the RNA molecule can bc morc important than its primary sequence for protein recognition. RNA helices with internal loops or bulges typically occur in regions of RNA involved in protein interactions, suggesting that such structures arc a common strategy for RNA-protcin recognition (see, for example, Draper, 1989, Trends Biochem. Sci. 14:335-338).

In various aspects of the invention, compounds are assayed for their ability to interfere with the interaction of a ribosomal protcin and an RNA target molecule. Such an RNA target comprises an RNA binding site for thc ribosomal protein. The RNA binding site can be either thc cognate binding site of the r-protcin or a structurally analogous RNA, such that the interaction of the structurally analogous RNA with thc r-protein is functionally similar to the interaction of the r-protein's cognate RNA binding site with the r-protein. Thus, a compound that interferes the interaction of a r-protcin with the analog will also interfere with thc r-protcin's activity in its natural context. For example, an RNA binding site of the invention may comprise the sequence of the mRNA recognition site of a

repressor r-protein, the rRNA binding site of a primary binding protein, or alternatively, an analog RNA binding site which is structurally and functionally analogous to a cognate RNA binding site.

The preferred RNA binding site of the present invention is an S8 protein RNA binding site. S8 protein is the repressor r-protein of the *spc* operon, and also a primary binding r-protein. A conserved secondary structure present in both mRNA and rRNA is recognized by S8 proteins of *E. coli* and other bacteria, such as *H. influenza*. This conserved stem is depicted in Figure 3. The precise primary sequence of the RNA in this region may vary, and many such variant base-pairings that preserve the structure of the S8 recognition site are possible. The preferred RNA binding sites of the invention are, therefore, the S8 mRNA binding site, the S8 rRNA binding site, or a hybrid RNA binding site that contains a variation of the mRNA and rRNA sequences, such that the hybrid's secondary structure forms an S8 recognition site. All such RNA binding site hybrids, analogs, and mutants are suitable for use in the methods of the present invention. The design and construction of such a variant S8 RNA binding site is described in detail in the example in Section 6.

In one embodiment, the invention provides translation assays to evaluate the ability of a test compound to interfere with the binding of an r-protein to an RNA binding site on a reporter mRNA molecule. Normally, binding of the r-protein to its RNA binding site on the mRNA blocks the translation of the reporter mRNA. However, in the presence of a test compound that interferes with the interaction of an r-protein and its RNA binding site, translation of the reporter mRNA is increased, *i.e.*, derepressed, which in turn leads to an increased production of the reporter. Depending on the reporter, a signal is generated which can be measured by the methods of the invention. By comparing the activity of the reporter in the presence of a test compound with its activity in the absence of the test compound (the controls), a test compound that is capable of interfering with binding of the r-protein can be identified. A compound that produce a positive result in this assay is expected to perturb the coordinated synthesis of ribosomal proteins *in vivo*. As a result, the process of ribosome assembly in the cell may also be disrupted, leading to the inhibition of bacterial cell growth.

The reporter mRNA useful in the methods of the present invention may be produced by a variety of methods known in the art. The reporter mRNA can be produced by transcribing a DNA molecule, herein referred to as a reporter gene construct, which comprises a bacterial promoter operably linked to a reporter gene, wherein the reporter gene comprises DNA sequences corresponding to the RNA binding site for the respective r-protein.

The methods of the invention can be carried out *in vivo* in a test cell or in an *in vitro* reaction. A test cell of the invention comprises the reporter gene construct and all the factors necessary for transcription and translation of the reporter gene. The r-protein may be under the control of an inducible promoter to allow regulation of the amount of r-protein present in a test cell. In an *in vitro* reaction, the reporter mRNA is incubated with the r-protein, a test compound and the factors necessary for translation of the reporter mRNA. Alternatively, a reporter gene construct can be used, if the appropriate enzymes and factors are provided to produce the reporter mRNA *in vitro*. The factors necessary for transcription and/or translation may be supplied in the form of a cell extract. Purified r-proteins, translation factors, and transcription factors also can be used as a supplement to a cell extract. Accordingly, the invention also encompasses reporter mRNAs, reporter gene constructs, test cells comprising reporter mRNAs and/or reporter gene constructs, and reaction mixtures comprising reporter mRNAs and/or reporter gene constructs, useful in the various translation assays.

In yet another embodiment, the invention provides functional assays that evaluate the ability of a test compound to block the binding of an r-protein to an RNA binding site on an mRNA. The assays are based on measuring, in the presence of a test compound, the increase in growth of recombinant test cells that have been engineered to overexpress an r-protein. Normally, as a result of the overexpression, the recombinant cell exhibits a phenotype of retarded growth under a particular culture condition. In the presence of an effective test compound, the excess r-protein will be blocked from its binding site on the mRNA, resulting in a more rapid growth rate of the test cells. A test compound capable of interfering with the binding of an r-protein to its specific RNA binding site on an mRNA molecule can thereby be identified by an increase in the growth rate of the recombinant test cells.

In another embodiment, the invention provides binding assays that measure the ability of a test compound to interfere with the binding of a ribosomal protein to an RNA binding site. The binding assays are based on measuring the amount of RNA or protein capable of forming RNA-protein complexes in the presence of a test compound. The binding assays use an RNA target molecule comprising at least one RNA binding site specific for a repressor r-protein or primary binding r-protein. In the assays of the invention, an r-protein and an RNA target are incubated in the presence of a test compound. Normally, the ribosomal protein will bind to the RNA binding site(s) on the target RNA molecule resulting in the formation of RNA– protein complexes. However, if a test compound capable of blocking the binding of the r-protein to the RNA binding site is present, fewer or no complexes are formed. The effect of the test compound on the specific binding of an r-protein to an RNA binding site can be evaluated by measuring the amount of RNA–protein complexes formed during the incubation, or by measuring the amount of residual unbound target RNA or residual unbound r-protein left at the end of the incubation. Inhibition of binding of an r-protein to an RNA binding site is indicated by a decrease in the formation of the RNA-protein complexes, or an increase in either the residual r-protein or the residual RNA, relative to the appropriate controls which are not contacted with the test compound.

In various embodiments of the invention, to facilitate detection and quantitation, at least one of the components of a binding reaction, *i.e.*, either the RNA target or the ribosomal protein, or both, is labeled. The RNA target may be labeled by any methods known in the art for labeling nucleic acid molecules, such as, but not limited to, incorporation of radioactive nucleotides during synthesis or chemical modification of the RNA molecule, provided that the method does not significantly affect the specific binding of the r-protein. Methods for labeling the RNA target are described in detail in Section 5.3.6, *infra*. The repressor r-protein or primary binding r-protein can be labeled by any methods known in the art for labeling a protein or polypeptide, such as, but not limited to, iodination, phosphorylation, derivatization with polyfunctional linker molecules, etc., provided the method does not significantly affect the protein's specific interaction with the RNA binding site. Methods for labeling a ribosomal protein are described in Section 5.3.6, *infra*.

In another embodiment, to facilitate detection and quantitation, the binding assays of the invention employ components that comprise an affinity tag which allows a tagged component to be conveniently separated from the untagged components in a binding reaction. Typically, this can be accomplished by tagging one of the components, either the ribosomal protein or the RNA target molecule, with an affinity compound. The affinity tag can be captured by a binding partner of the affinity tag, which is immobilized onto a solid phase surface. After the binding reaction is complete, RNA-protein complexes comprising the affinity-tagged component, and excess unbound tagged component, can be captured onto the solid phase surface for measurement, by methods well known in the art.

Alternatively, an agent that specifically binds the ribosomal protein or the RNA target molecule may be used. Such an agent, *e.g.*, an antibody against the ribosomal protein, or a nucleotide sequence complementary to the RNA target molecule, can be immobilized on a solid phase surface to capture the products of the binding reaction. For example, the target RNA can comprise, in addition to the RNA binding site for a specific ribosomal protein, a nucleotide sequence complementary to the nucleotide sequence of another nucleic acid molecule which is immobilized onto a solid phase. The target RNA thus can be captured onto the solid phase by hybridization to the immobilized nucleic acid molecule. Use of antibodies against the ribosomal proteins or against nucleotide sequences complementary to the target RNA, provided that their interaction with their protein or RNA binding partner does not significantly alter the interaction between the r-protein and the RNA binding site, are within the scope of the invention.

The invention also encompasses RNA target molecules, gene constructs encoding the RNA targets, and recombinant cells containing the target RNAs and/or gene constructs encoding these RNAs. The invention further encompasses ribosomal proteins containing an affinity tag, gene constructs encoding ribosomal proteins containing an affinity tag, and recombinant cells comprising a gene construct encoding a ribosomal protein containing an affinity tag.

The compounds tested in the methods of the invention can be obtained from a wide variety of sources including collections of natural products in the form of bacterial, fungal, plant and animal extracts; and synthetic chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide

variety of chemical structures. In addition, natural products or known antibiotic compounds may be subjected to random or directed chemical modifications to produce derivatives and structural analogs for use as test compounds in the invention. Usually, various predetermined concentrations are used for screening such as 0.001 μm, 0.01 μm, 0.1 μm, 1.0 μm, 10 μm, and 100 μm. Typically, these compounds have a molecular weight of more than about 50, but less than about 3,000, and preferably less than 1,000.

Test compounds that score positive in the screening assays of the invention are putative agents that interfere with the binding of regulatory r-proteins to RNA, and are useful as leads for the development of therapeutic agents for the treatment of infectious diseases. Drugs based on such agents are likely to be both very effective in controlling growth in a broad spectrum of bacteria as well as safe for use, causing minimal side effects in the treated subject.

In various embodiments of the invention, the contacting of a test compound may be effected in any vessel and by any means, such as wells or disks impregnated with a solution or suspension of a test compound. Standard protocols, such as serial dilution, may be used. The amount of time allowed for the test compound to interfere with the function of a ribosomal protein in a test cell may be determined empirically, such as by running a time course and monitoring the accumulation of reporter molecule as a function of time.

For clarity of discussion, the invention is described in the subsections below by way of example in *E. coli*. However, the principles may be applied to other bacteria that contain ribosomal proteins which are functionally equivalent to those in *E. coli*.

5.1 Ribosomal Proteins and RNA Binding Sites

Described herein are the ribosomal proteins and RNA molecules useful in the methods of the invention. In the translation assays and the binding assays of the invention of the invention, either a repressor ribosomal protein or a primary binding ribosomal protein serves as a target protein for the test compounds. It should be noted that certain repressor r-proteins in addition to binding to mRNA, also bind to rRNA, and likewise, certain primary binding r-proteins in addition to binding to rRNA, also bind to mRNA.

Amino acid sequences and nucleotide sequences of naturally occurring r-proteins are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Nucleic acids encoding the r-proteins and fragments thereof are provided, as well as nucleic acids complementary to and capable of hybridizing to such nucleic acids. The complete nucleotide sequences of many r-proteins and rRNAs are known in the art. Non-limiting examples of such sequences are those of *E. coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Mycoplasma genitalium*, and *Chlamydia trachomatis* (Blattner et al.; 1997, *supra*; Tomb et al., 1997, *supra*; Fleischmann et al., 1995, Science, 269:496-512; Fraser et al., 1995, Science 270:397-403; Stephens et al., Science, Oct. 23, 1998).

Although the ribosomal proteins of *E. coli* are used herein for purpose of description of the invention, it is contemplated that the invention encompasses the use of equivalent ribosomal proteins of other bacteria. The *E. coli* ribosomal proteins and their equivalents in other bacteria are expected to share a high degree of sequence and structural similarity. Such equivalent ribosomal proteins, and their genes in other bacteria can be identified by techniques commonly known in the art. Non-limiting examples of such techniques include polymerase chain reaction using known or degenerate sequences as primers, immunoassays using cross-reacting antibodies, or hybridization assays using the *E. coli* gene as a probe.

The DNA encoding an r-protein of *E. coli* or another bacteria, herein referred to as an r-protein gene, may be obtained by standard procedures known in the art, such as by DNA amplification from DNA prepared from cells or from cloned DNA (e.g., a DNA "library"). Likewise, genes encoding ribosomal RNA from *E. coli* and other bacteria, herein termed rRNA genes, can also be obtained by such methods. Whatever the source, the r-protein gene or rRNA gene should be molecularly cloned into a suitable vector for propagation of the gene.

In a preferred embodiment for obtaining an r-protein or rRNA gene, polymerase chain reaction (PCR) is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, e.g., by use of a thermal cycler and Taq polymerase (Gene Amp™). The DNA being amplified can include cDNA or genomic DNA from any species of bacteria. Oligonucleotide primers representing known nucleic acid sequences of related r-proteins can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the r-protein gene that is highly conserved between regulatory r-proteins of different species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known r-protein nucleotide sequence and the nucleic acid homolog being isolated. For cross-species hybridization, low stringency conditions are preferred. For closely related species, moderately stringent conditions are preferred. After successful amplification, the sequence encoding a r-protein may be cloned and sequenced. If the size of the coding region of the regulatory r-protein gene being amplified is too large to be amplified using a single set of PCR primers, several sets of PCR primers can be used covering the entire gene, preferably with overlapping regions, and the appropriate fragments of the PCR products ligated together to form the entire coding sequence. Alternatively, if a segment of an r-protein gene is amplified, that segment may be cloned, and utilized as a probe to isolate a complete cDNA or genomic clone.

In another embodiment, the r-protein gene or rRNA gene may be isolated by molecular cloning from genomic DNA library of *E. coli* or another species of bacteria. DNA fragments can be generated from cellular DNA and cloned to form a genomic library. Since the sequences encoding r-proteins of *E. coli* are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to the labeled probe (Benton & Davis, 1977, Science 196:180; Grunstein & Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available.

Other methods for isolating the r-protein genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA that encodes the r-protein. For example, RNA for cDNA cloning of the r-protein gene can be isolated from cells which express the regulatory r-protein. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to the r-protein is available, the r-protein may be identified by binding of labeled antibody to clones that synthesize the putatively regulatory r-protein.

Other specific embodiments for the cloning of a nucleotide sequence encoding a r-protein, are presented as examples but not by way of limitation, as follows:

In a specific embodiment, nucleotide sequences encoding r-protein of another species of bacteria can be identified and obtained by hybridization with a probe comprising nucleotide sequence encoding the *E. coli* r-protein under conditions of low to medium stringency.

By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (e.g., as employed for identifying genes within the same family).

The r-protein or rRNA gene can be inserted into an appropriate cloning vector and introduced into host cells so that many copies of the gene sequence are generated. A large number of vector-host systems known in the art may be used such as,

but not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene).

The above methods are not meant to limit the methods by which clones of regulatory r-protein may be obtained or propagated.

5.1.1 Repressor R-Proteins and mRNA Binding Sites

The repressor r-proteins can be used to inhibit (or *repress*) translation of an mRNA comprising an RNA binding site in the translation assays of the invention, and can also be used to bind an RNA binding site in the physical binding assays of the invention. Such repressor r-proteins include but are not limited to S1, S4, S7, S8, S15, S20, L1, L4, L10 and L20. The term "repressor ribosomal protein" or "repressor r-protein" encompasses the entire protein, functional fragments and derivatives thereof, including those that are purified from bacterial cells and those produced recombinantly in a cell or in an *in vitro* reaction. The fragments or derivatives useful in the methods of the invention are functional in binding to the RNA binding site on a reporter mRNA thereby inhibiting translation of the reporter mRNA. Such fragments or derivatives are known, or can readily be identified by assaying their effect on translation of the reporter mRNA in comparison to that of the entire protein. Such repressor r-protein fragments and derivatives can be prepared by any of a variety of methods known in the art, including recombinant expression of a gene encoding a truncated repressor r-protein or isolation of proteolytic fragments of the repressor r-protein. Preferred repressor r-proteins of the invention are described herein.

As shown in Figure 1, the genes encoding ribosomal proteins are organized into ten operons in *E. coli*, known as *spc*, *α(S4)*, *S10*, *S15*, *str*, *S20*, *S1*, *L11*, *L20*, and *rif* (Zengel & Lindahl, 1994, Prog. Nucl. Acid Res.; 47: 331-370). These operons also include other *E. coli* genes, such as the gene encoding RNA polymerase subunits and ancillary proteins involved in protein synthesis. A common feature of these operons is that in each operon, one of the ribosomal protein products acts to inhibit expression of a contiguous set of genes within the operon. The ribosomal protein that acts as such a negative autogenous regulator is the repressor r-protein of the invention.

Without being bound by any theory, translation inhibition may result from the ribosomal protein directly blocking the ribosome's access to the mRNA by binding of

the ribosomal protein to the ribosomal binding site, by altering the conformation of the mRNA such that the ribosomal binding region is inaccessible by the ribosome, or by interfering with a subsequent stage of the translation process.

The organization of the *E. coli* the r-protein genes and other genes within each operon is shown in Figure 1. The ten translation repressor r-proteins (underlined in Figure 1) and their mRNA recognition sites (double asterisks in Figure 1) are discussed in detail herein. The nucleotide positions of the mRNA binding sites provided herein for each repressor r-protein corresponds to the numbering scheme used in the specific references cited, which are each incorporated herein in their entireties.

S8 is one of the most preferred target ribosomal protein for use in the methods of the invention. S8 is the fifth gene product of the *spc* operon, which encodes 11 r-protein genes (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616; GenBank Accession No. X01563). As shown in Figure 2, ribosomal proteins equivalent to S8 in other bacteria are also known in the art, and share a high degree of sequence similarity with each other. The use of such equivalents of S8 in screening assays, and similarly, for equivalents of other repressor r-proteins are contemplated. S8 regulates translation of its mRNA by binding to a site at the beginning of the third gene of the operon, L5, between residues +22 to +81 within the double-stranded stem of a conserved stem-loop secondary structure (Gregory et al., 1988, J. Mol. Biol. 204:295-307). Figure 3 shows the secondary structure of the S8 binding site on the *spc* mRNA of *E. coli* and *H. influenzae*, and their similarities to the secondary structure of its binding site on 16S rRNA, as discussed in Section 5.1.2, *infra*.

The L4 protein (GenBank Accession No. X02613; Zurawski & Zurawski, 1985, *supra*), the product of the fourth gene of the S10 operon, regulates both the transcription and the translation of the S10 operon by binding to a site on the S10 leader sequence of the mRNA. Two adjacent hairpin loop structures formed by RNA base pairing of residues located between positions 84 and 145 (named HE) and 146 and 192 (named HG) are required for L4-mediated translational control (Zengel & Lindahl, 1994, *supra*). According to structure probing studies, L4 binds to a single-stranded region at approximately position 145 between the two hairpin loop structures HE and HG. The S10 leader sequences of several other enterobacteria have also been shown to be highly

homologous to that of *E. coli* (Shen, 1991, Ph.D. thesis, University of Rochester, New York).

S4 protein (Bedwell et al., 1985, Nucl. Acids Res. 13:3891-3903; GenBank Accession No. X02543), the third gene of the α operon, regulates translation by binding to the leader sequence of the mRNA transcribed from the operon. The S4 binding site encompasses bases -84 to +40 of the α mRNA. The mRNA of this region forms two intertwined pseudoknots composed of four helices and six single-stranded regions, including the ribosome binding site, and the proximal 40 bases of the S13 gene (Deckman & Draper, 1987, J. Mol. Biol. 196: 313-332) .

The S7 protein, the second gene product of the *str* operon, regulates its own expression and the expression of the downstream proximal gene product, elongation factor-G, but has little effect on other genes in the operon. S7 binds to the region between the genes for S12 and S7 (Post & Nomura, 1980, J. Biol. Chem. 255:4660-4666; GenBank Accession No. ES3149).

The S15 protein (Regnier et al., 1987, J. Biol. Chem. 262:63-68; GenBank Accession No. J02638) autoregulates translation from the S15 operon by binding to the ribosome binding site region, stabilizing a pseudoknot structure, instead of an alternative hairpin structure that is required for translation initiation (Philippe et al., 1990, J. Mol. Bio. 211:415; Portier et al., 1990, J. Mol. Bio. 211:407-414; Philippe et al., 1990, Proc. Natl. Acad. Sci. U.S.A., 90:4394).

L1 is the second gene of a two r-protein operon, the L11 operon. L1 binds to region from -52 to +9 in the L11 leader, overlapping the ribosome binding site, and encompassing a structure homologous to the L1 binding site in 23S RNA (Kearney & Nomura, 1988, Mol. Gen. Genet. 210:60).

The L10 protein is the first gene product of the *rif* operon, which encodes four r-proteins as well as two RNA polymerase subunits (Post et al., 1979, *supra*; GenBank Accession No. J01678). L10 forms a complex with L12, and the L10-$(L12)_4$ complex autoregulates this operon by binding to the leader sequence at positions -120 to -160, within a double-hairpin loop structure that spans a region from -212 to -80 upstream from the initiation codon of the L10 gene (Climie & Frieson, 1987, J. Mol. Biol, 198: 371).

The L20 protein is part of a complex transcription unit with genes for L35 and two translation factors (Fayat et al., 1983, J. Mol. Biol. 171:239-261; GenBank Accession No. M10423). L20 is the repressor of its own gene and the gene for L35 (Lesage et al., 1992, J. Mol. Biol., 228:366). A region of the upstream *infC* gene, which encodes initiation factor-3, is known to be essential (Lasage et al., 1990, J. Mol. Biol., 213:465).

The S20 protein (Mackie, 1981, J. Biol. Chem. 256:8177-8182; GenBank Accession No. X04382) is encoded by a monocistronic operon and regulates its own translation. S20 regulation is thought to occur by binding of S20 to the 30S::S20-mRNA tertiary complex (Parsons et al., 1988, J. Bacteriol. 10: 2485) or by a mechanism involving repression ribosomes containing S20 (Gotz et al.,1990, Biochem. Biophys. Acta 1050:93).

S1 protein, the only translational regulatory protein that is not a primary binding protein, is the only protein encoded by the S1 operon.. The S1 protein has a general and weak affinity for RNA, particularly oligo(U)-containing sequences (Boni et al., 1991, Nuc. Acids Res. 19:155).

Due to the degeneracy of the genetic code, the term "ribosomal protein gene sequence" refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the ribosomal protein. Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, for creating/deleting restriction sites, or for adding affinity tags. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill et al., 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho et al., 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar et al., 1990, Biotechniques, 8:404-407), etc. Modifications can be confirmed by DNA sequencing.

In addition, modified regulatory r-proteins can be synthesized comprising a peptide tag which facilitates recovery and purification. The peptide tag can be associated with any portion of the repressor r-protein, so long as such association does not alter the binding of the r-protein with the RNA binding site. In various embodiments, such a fusion

protein can be made by ligating a regulatory r-protein gene sequence to the sequence encoding the peptide tag in the proper reading frame. Care should be taken to ensure that the modified gene remains within the same translational reading frame, uninterrupted by translational stop signals.

A variety of peptide tags known in the art may be used in the modification of a regulatory r-protein, such as but not limited to the polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan et al., 1987, Gene 67:21-30), and various cellulose binding domains (U.S. patent 5,496,934; 5,202,247; 5,137,819; Tomme et al., 1994, Protein Eng. 7:117-123), etc. Other possible peptide tags are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemaglutinin (HA) epitope. Other peptide tags are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner, which is preferably immobilized and/or on a solid phase surface. As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the above-mentioned peptide tags, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the peptide tags and reagents for their detection and isolation are available commercially.

DNA sequences encoding desired peptide tags which are known or readily available from libraries or commercial suppliers are suitable in the practice of this invention. Methods for obtaining regulatory r-protein sequences described in Section 5.1.1 can also be applied to obtain sequences encoding a peptide tag.

5.1.2 Primary Binding R-Proteins and rRNA Binding Sites

The r-proteins that can be used in the present methods include the primary binding ribosomal proteins S4, S7, S8, S15, S17, S20, L1, L2, L3, L4, L7/12, L10, L11, L15, L20, L23 and L24. Such primary binding r-proteins can bind to specific sites in rRNA in the absence of binding by other r-proteins, a step required for ribosome assembly *in vivo*.

A primary binding r-protein, or a peptide fragment comprising an RNA binding site thereof, can be obtained by purification of an r-protein or fragment from native sources, such as bacterial extracts. For example, ribosomal proteins, and fragments thereof, can be isolated from total extracts of cellular proteins, by methods well known in the art (Ausubel, *supra*). Alternatively, an r-protein or fragment may be obtained by molecular cloning and expression of ribosomal protein genes. Various methods can be used to obtain the coding region of such ribosomal protein gene sequences, including, but not limited to, DNA cloning, DNA amplification, and synthetic methods, as will be appreciated by those skilled in the art.

The nucleotide sequences of non-limiting examples of *E. coli* ribosomal protein genes that can be expressed by methods of the invention are published as follows: S4, GenBank Accession No. X02543 (Bedwell et al., 1985, Nucl. Acids Res. 13:3891-3903); S7, GenBank Accession No. ES3149 (Post & Nomura, 1980, J. Biol. Chem. 255:4660-4666); S8, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616); S15: GenBank Accession No. J02638 (Regnier et al., 1987, J. Biol. Chem. 262:63-68); S17, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res.13:4521-4526); S20, GenBank Accession No. X04382 (Mackie, 1981, J. Biol. Chem. 256:8177-8182); L1: GenBank Accession No. J01678 (Post et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1697-1701); L2, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res. 13:4521-4526); L3, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, *supra*); L4, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, *supra*); L7/12, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L10, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L11, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L15, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616); L20, GenBank Accession No. M10423 (Fayat et al., 1983, J. Mol. Biol. 171:239-261); L23, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res. 13:4521-4526); and L24, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616). Due to the degeneracy of the genetic code, the term "ribosomal protein gene sequence" refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the ribosomal protein.

The most preferred primary binding proteins for use in the methods of the present invention is S8. The primary binding proteins S8 and S15 interact with bases within the central domain of the 16S rRNA molecule (Gregory et al., 1984, J. Mol. Biol., 178:287-302). The S8 protein binds to rRNA at approximately 588-606 and 632-651 at the base of a stem-loop hairpin located in the central domain of 16S rRNA (Svensson et al., 1988, J. Mol. Biol. 200:301-308; Cerretti et al., 1988, J. Biol. Chem. 204:309; Gregory et al., 1988, 204:295-307). S8 also interacts with positions 573-575, 583, 812, 858-861 and 865 in this general region. S15 interacts with nucleotides in the 655-672 and 734-751 stem, also located in the 16S central domain.

S4 protein (Bedwell et al., 1985, Nucl. Acids Res. 13:3891-3903; GenBank Accession No. X02543) binds to three helices of 16S rRNA approximately at positions 73-95, 372-388, and 455-476, as determined by genetic and footprint analysis (Stern et al., 1986, 192:101; Sapag et al., 1990, Biochem. Biochem. Acta 229:609).

The S15 protein (Regnier et al., 1987, J. Biol. Chem. 262:63-68; GenBank Accession No. J02638) binds to 16S rRNA at a hairpin between residues 655 and 752.

L4 protein binds 23S rRNA in 50S subunits at a single-stranded region within a hairpin loop structure formed by base-pairing between 265 and 427 of 23S rRNA called domain I, and about 300 bases from the 5' end (Zengel & Lindhal, 1993, Nucl. Acids Res. 21: 2429; Zengel & Lindhal, 1991, Biochimie 73:719). L4 is directly cross-linkable to a sequence GCGAUACA located at approximately position 280 at the top of this domain (Gulle et al, 1988, Nucl. Acids Res. 16:815).

The L10/L12 r-protein complex binds 23S rRNA at position 1045 to 1055 within a hairpin loop structure that spans the region from 1030 to 1124 (Egebjerg et al., 1990, J. Mol. Biol. 213: 275).

5.2 Assays Based on Translation

The present invention provides *in vivo* and *in vitro* translation assays for evaluating the ability of a test compound to interfere with or block the binding of an r-protein to its RNA binding site. The methods of the invention are based on allowing an r-protein to bind specifically to its RNA binding site on an mRNA encoding a reporter

(reporter mRNA), in the presence of a test compound, and determining the expression of the product encoded by the reporter mRNA .

The *in vivo* translation assay of the invention involves a test cell which comprises a reporter gene construct and all the factors necessary for transcription and translation of the reporter gene. Detailed description of reporter gene constructs and test cells of the invention are described in Section 5.2.1 and 5.2.2, respectively.

The *in vitro* assays of the invention are carried out using a reporter mRNA, an r-protein, a test compound, and other factors necessary for translation of the reporter mRNA. Such factors necessary for transcription and/or translation may be supplied in the form of a cell extract.

The *in vivo* assays and *in vitro* assays have their advantages and disadvantages. Generally, *in vitro* assays are more expensive since active cell extracts containing labile ingredients are necessary. However, it might be advantageous to use an *in vitro* assay where a compound is so potent as to be lethal to the host cell in an *in vivo* assay. Such compounds can be readily tested *in vitro*. It might also be desirable to carry out the assays *in vitro* if methods for manipulating genetic material in the target microbial organism are not well developed, or if it is hazardous to use pathogenic bacteria as test cells.

5.2.1 Reporter Messenger RNA

The term "reporter messenger RNA" or "reporter mRNA" as used herein refers to an RNA molecule comprising a specific RNA binding site for a ribosomal protein which is functionally associated with any ribonucleotide sequence that encodes a detectable polypeptide. When used in an *in vivo* assay, the reporter molecule, *i.e*, the translation product of the reporter mRNA, should be easily detectable and distinguishable from other proteins present in test cells. Preferably, the reporter mRNA encodes a protein that is readily detectable either by its presence, or by its activity that results in the generation of a detectable signal. A reporter mRNA is used in the invention to monitor and report the ability of a test compound to block or interfere with the interaction of an r-protein with its RNA binding site. The term "functionally associated" as used herein refers to an arrangement wherein the ribonucleotide residues to which a specific r-protein binds and the coding region of a detectable polypeptide are joined and positioned such that the translation

of the coding region is under the control of the r-protein. That is, the specific binding of the r-protein to the reporter mRNA of the invention leads to repression of its translation. In many cases, the specific binding site also comprises the initiation codon fused in frame to the 5' end of the coding region of the detectable polypeptide.

The reporter mRNA comprises an RNA binding site. The RNA binding site may be either the cognate binding site of a repressor r-protein, the cognate binding site of a primary binding r-protein, or a segment of RNA having a similar secondary structure, such that the interaction of the structurally analogous RNA with the r-protein is similar to the interaction of the r-protein's cognate RNA binding site with the r-protein. For example, the RNA binding site may comprise the sequence of the mRNA recognition site of a repressor r-protein, the rRNA binding site of a primary binding protein, or alternatively, an analog RNA binding site that is functionally and structurally similar to the cognate mRNA or rRNA binding site. The nucleotide sequences of the mRNA binding sites for each repressor r-protein are described in Section 5.1.1, and the binding sites on rRNA for primary binding r-proteins are described in Section 5.1.2. Any of the specific binding sites as described in Section 5.1.1 and their equivalents in other bacteria can be used to prepare a reporter mRNA for use in a translation assay of the invention.

The reporter mRNA may additionally comprise a ribosome binding site, a terminator site, as well as additional open reading frames, if desired. Where the cognate mRNA binding site for a repressor protein is being used, the natural mRNA sequence may contain the required ribosome binding sequences, initiation codons and the like. However, where rRNA binding site or analog RNA binding site is being used, an initiation codon and Shine-Delgarno ribosome binding site should be added for translation initiation. By way of an example, a reporter gene construct comprising an analog S8 RNA binding site (a hybrid RNA binding site designed from the sequences of the mRNA binding site and the rRNA binding site of the repressor r-protein S8) fused to the Renilla luciferase gene is provided (see Section 6 and Figure 6).

The term "reporter gene" as used herein refers to a DNA molecule which produces a translatable reporter mRNA of the invention upon transcription under the appropriate conditions. The reporter gene may be inserted into a recombinant expression vector. A "reporter gene construct" comprises a reporter gene and other nucleotide

sequences required for transcription of the reporter gene, and/or sequences for maintenance of the construct in a test cell. The term encompasses a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of reporter gene sequences. Such reporter gene constructs of the invention are preferably plasmids. Hence, a reporter gene construct may comprise a promoter, one or more origins of replication, and one or more selectable markers which allow phenotypic selection of recombinant cells. The reporter gene construct may also provide unique or conveniently located restriction sites to allow severing and/or rearranging portions of the DNA inserts in a reporter gene construct. Typically, a reporter gene construct is created in the course of constructing the fusion of an r-protein binding site and the coding region of the reporter. A reporter gene construct is used in the invention to generate large amounts of reporter mRNA in test cells or *in vitro*. More than one type of reporter gene may be inserted into the construct such that the results may be assayed by different means. Standard molecular biology techniques can be used to construct reporter gene constructs of the invention.

The coding region of a reporter mRNA comprises a nucleotide sequence that encodes a reporter molecule which is capable of directly or indirectly generating a detectable signal. In many cases, the coding region of a reporter mRNA may also comprise a nucleotide sequence that is a part of the specific r-protein RNA binding site. Some of such nucleotide sequences may encode the amino terminal of a ribosomal protein the synthesis of which is under the control of the repressor r-protein.

Generally, although not necessarily, the reporter mRNA encodes a detectable protein that is not otherwise produced by the test cells. Many such detectable proteins and their coding regions have been described, and some are commercially available for the study of gene regulation. See, for example, Alam & Cook, 1990, Anal. Biochem. 188:245-254, the disclosure of which is incorporated herein by reference.

For convenience and efficiency, enzymatic reporters and light-emitting reporters are preferred for the screening assays of the invention.. Accordingly, the invention encompasses histochemical, colorimetric and fluorometric assays.

A variety of bioluminescent, chemiluminescent and fluorescent proteins can be used as light-emitting reporters in the invention. A preferred reporter for use in the present methods is Renilla luciferase. Other sources of luciferase, an enzyme that requires

cofactor(s) to emit light, are also well known in the art, including but not limited to, the bacterial luciferase (*luxAB* gene product) of *Vibrio harveyi* (Karp, 1989, Biochim. Biophys. Acta 1007:84-90; Stewart et al. 1992, J Gen Microbiol, 138:1289-1300), and the luciferase from firefly, *Photinus pyralis* (De Wet et al. 1987, Mol. Cell. Biol. 7:725-737).

Another type of light-emitting reporter, which does not require substrates or cofactors, includes but is not limited to the wild type green fluorescent protein (GFP) of *Victoria aequoria* (Chalfie et al. 1994, Science 263:802-805), and modified GFPs (Heim et al. 1995, Nature 373:663-4; PCT publication WO 96/23810). Transcription and translation of this type of reporter gene leads to the accumulation of the fluorescent protein in test cells, which can be measured by a device, such as a fluorimeter, or a flow cytometer. Methods for performing assays on fluorescent materials are well known in the art and are described in, *e.g.*, Lackowicz, J.R., 1983, Principles of Fluorescence Spectroscopy, New York:Plenum Press.

A variety of enzymes may be used as a reporter which includes but are not limited to chloramphenicol acetyltransferase (CAT; Gorman et al., 1982, Mol Cell Biol, 2:1044; Prost et al., 1986, Gene 45:107-111), β-galactosidase (Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607), β-lactamase, β-glucuronidase and alkaline phosphatase (Berger et al., 1988, Gene 66:1-10; Cullen et al., 1992, Methods Enzymol; 216:362-368). The *E. coli lacZ* gene, a commonly used reporter gene encodes β-galactosidase, which is a very stable enzyme and has a broad specificity so as to allow the use of different chromogenic or fluorogenic substrates, such as but not limited to lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and fluorescein galactopyranoside (Molecular Probes, Oregon). See, Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607. Another commonly used reporter gene is the *E. coli* β-glucuronidase gene (GUS; Gallagher, 1992, in "GUS protocols", Academic Press) which can be used with various histochemical and fluorogenic substrates, such as X-glucuronide, and 4-methylumbelliferyl glucuronide. Transcription and translation of a reporter gene leads to production of an enzyme in test cells or in reactions. The amount of enzyme present can be measured via its enzymatic action on a substrate resulting in the formation of a detectable reaction product. The methods of the invention provides means for determining the amount of reaction product, wherein the amount of reaction product

generated or the remaining amount of substrate is related to the amount of enzyme activity. For some enzymes, such as β-galactosidase, β-glucuronidase and β-lactamase, fluorogenic substrates are available that allow the enzyme to covert such substrates into detectable fluorescent products (see, for example, U.S. Patent No. 5,070,012, and PCT Patent WO 96/30540).

Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, such as substrates and cofactors for reporter molecules that are enzymes; e.g., X-gal, lactose-tetrazolium medium. Such compositions are well known in the art. Components of a reporter regimen may be supplied to the test cells during any step of the screening assay.

Any antigenic peptide or protein that can be detected by an antibody can also be used as a reporter, for example, growth hormone (Selden et al., Mol. Cell Biol., 6:3173). To facilitate detection by antibody binding in immunoassays, antigenic reporter molecules that are secreted or attached on the test cell surface are preferred.

5.2.2 Test Cells

Bacterial cells useful in the present invention may be obtained from private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers. It is desirable to use bacteria that have been developed for drug screening processes, and for which conditions for growth, maintenance, and manipulations are known. The most preferred bacterial species that is useful as test cells is *Escherichia coli*. Other preferred bacterial species may include but not limited to *Bacillus subtilis*, and *Pseudomonas aeuroginosa*. *Escherichia coli* can be used as a model of many bacteria.

Test compounds that specifically interfere with the binding of an r-protein to mRNA in *E. coli* test cells are expected to have a similar inhibitory effect on the translation of the ribosomal RNA in other pathogenic species, such as but not limited to, *Vibrio* species, *Pseudomonas* species, *Acinetobacter* species, *Bordetella* species, *Campylobacter* species, *Haemophilus* species, *Neisseria* species and Enterobacteriaceae species, such as

Salmonella, Enterobacter, Klebsiella, Yersinia, Proteus, Serratia, and *Staphylococcus* species, *Streptococcus* species, *Corynebacterium* species, *Listeria* species and *Bacillus* species. It is also expected that positive test compounds will be effective as an antibiotic against multidrug-resistant strains of these pathogenic species, such as β-lactam-resistant strains of *E. coli*.

A test cell of the invention comprises the reporter mRNA as described in Section 5.2.1. A variety of methods for introducing the reporter mRNA can be used to prepare test cells of the invention. Most conveniently, the reporter mRNA is generated in a test cell by the use of a reporter gene construct which is introduced into a bacterial cell and propagated in progenies of the bacterial cell. Introduction of the reporter gene construct may be carried out by conventional techniques well known to those skilled in the art, such as transformation, conjugation, and transduction. For example, where the host is *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl could be used.

In addition to conventional chemical methods of transformation, the reporter mRNA or reporter gene constructs of the invention may be introduced into a host cell by physical means, such as by electroporation or microinjection. Electroporation allows transfer of the construct by high voltage electric impulse, which creates pores in the plasma membrane of the host and is performed according to methods well known in the art. Additionally, reporter mRNA or reporter gene construct can be introduced into host cells by protoplast fusion, using methods well known in the art.

The test cells which contain the reporter gene sequence and which express the reporter gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions (*e.g.*, resistance to antibiotics); (c) assessing the level of transcription as measured by the expression of reporter mRNA transcripts in the host cell; and (d) detection of the reporter gene product as measured by immunoassay or by its biological activity.

The test cells may be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the bacteria. However, conditions for

maintenance and growth of the test cell may be different from those for assaying candidate test compounds in the screening methods of the invention. Modified culture conditions and media are used to facilitate detection of the expression of a reporter molecule. Any techniques known in the art may be applied to establish the optimal conditions.

Test cell strains, cell cultures, cell lines generated by the above-described methods for the screening assays may be expanded, stored and retrieved by any techniques known in the art that is appropriate to the test cell. For example, the test cells of the invention can be preserved by stab culture, plate culture, or in glycerol suspensions and cryopreserved in a freezer (at -20°C to -100°C) or under liquid nitrogen (-176°C to -196°C).

To facilitate high throughput screening, the test cells can be cultured and assayed in an ordered array, such as multi-well plates. Typically, the individual cultures are inoculated and allowed to grow in the wells under the appropriate conditions. Manipulations of the cultures and fluid handling can be done with a multi-channel devices. Most of the transfers and manipulations can be automated and miniaturized, and performed by laboratory robots.

5.2.3 Test Cell Extracts

The term "test cell extract" as used herein refers to an extract of a bacterial cell that comprises at least the components of the translation system that is functionally capable of translating a messenger RNA with the appropriate signals *in vitro*. The appropriate signals include, at a minimum, the ribosome binding site (the Shine-Dalgarno sequence), and a suitably positioned initiation codon (about 8 to 14 bases from the ribosome binding site). The test cell extract may contain the target r-protein of the *in vitro* assay.

In various embodiments, the test cell extract of the invention is an extract of test cells of the invention as described above, which comprises components of a functional translation system as well as a functional transcription system. The reporter mRNA used in the *in vitro* assay is produced by the transcription system in the presence of a reporter gene construct.

5.2.4 *In Vivo* Translation Assays

Described herein are *in vivo* translation assays of the invention which are based on measuring the effect of a test compound on the translation of a reporter mRNA in a test cell. The test cell comprises a reporter mRNA, as described in Section 5.2.1, which comprises an RNA binding site specific for an r-protein of interest that regulates the translation of the reporter gene. Any of the repressor r-proteins or primary binding r-proteins described in Section 5.1.1 and 5.1.2 can be used in this method of the invention.

Generally, the screening assays comprise first, contacting a test cell with a test compound for a time period and under conditions sufficient to allow the test compound to enter the test bacterial cell and inhibit the activity of a ribosomal protein. For example, the test cells may be grown in culture to reach a certain growth phase or density, and then exposed to a test compound. Alternatively, the test cells may be grown continuously in the presence of a test compound. Standard methods of culture can be used to grow the test cells of the invention, including but not limited to plate culture, liquid culture, etc.

If the test compound is capable of interfering with the repressor activity of the r-protein of interest, such as by blocking its binding to the reporter mRNA, translation of the reporter mRNA is derepressed. As a result, production of the reporter molecule is increased, which, in turn, can lead to an increase in signal. An increase in the signal in the presence of a test compound, relative to the control reaction in which the test cells were not contacted with the test compound, indicates that the test compound is capable of interfering with or blocking binding of the r-protein to the RNA binding site. Any method known in the art for detecting or measuring translation of the reporter mRNA *in vivo* can be used in this invention. Alternatively, the translation of the reporter mRNA can be assayed *in vitro*, for example, by isolating a sample of the test cells and measuring the activity or concentration of the reporter molecule. Since translation of the reporter mRNA leads to the biosynthesis of the reporter molecule in the test cell, the method of the invention encompasses the detection and measurement of the amount of reporter molecule, or the detection and measurement of the signal generated by the reporter molecule.

5.2.5 *In Vitro* Translation Assay

The *in vitro* translation assay of the invention is based on the same principle as the *in vivo* translation assays except that the interactions between the preselected ribosomal protein of interest and the test compound, and the translation process itself, are carried out not within a live cell, but in a reaction vial or microtiter plate, or any suitable container. Since the translation process is performed *in vitro*, all the necessary translation factors, cofactors, and tRNAs charged with amino acids must be provided. Such ingredients of the *in vitro* translation assays can easily be provided by using an extract of the appropriate bacterial cells. Depending on the ribosomal protein and the bacterial species of interest, it may be preferable to supplement the *in vitro* reaction with purified ingredients which can be obtained from native bacterial cells or from recombinant expression of the factor if available. In some cases, it may be advantageous to add an excess of the r-protein of interest to enhance the sensitivity of the assay.

In this assay, the r-protein is contacted with a test compound for a time period and under condition sufficient to allow the test compound to inhibit the activity of the ribosomal protein. The contacting of the r-protein with the test compound can be performed prior to mixing the ingredients of the translation reaction with the r-protein. Alternatively, the test compound and the r-protein can be added simultaneously to the translation extract. Standard conditions of buffer, temperature, etc, for *in vitro* translation can be used. Techniques known in the art to calibrate and optimize the reaction conditions may be applied. Any method known in the art for detecting or measuring translation of the reporter mRNA in vitro can be used, including methods for detecting or measuring signal generated by the reporter molecule.

5.2.6 Fast-growth Assay

In another embodiment, the invention provides a screening assay (the fast-growth assay) that uses a bacterial test cell that contains a nucleic acid sequence encoding a repressor ribosomal protein or a fragment thereof, operably linked to a promoter which is expressible in the cell such that the repressor ribosomal protein is produced in the cell.

The fast growth assay that is an assay is based on measuring, in the presence of a test compound, the increase in growth of recombinant test cells that have been

engineered to overexpress an r-protein. Normally, as a result of the overexpression of a repressor r-protein, the recombinant cell exhibits a phenotype of retarded growth under a particular culture condition. In the presence of an effective test compound, the excess r-protein will be blocked from its binding site on the mRNA, resulting in a more rapid growth rate of the test cells. A test compound capable of interfering with the binding of an r-protein to its specific RNA binding site on an mRNA molecule can thereby be identified by an increase in the growth rate of the recombinant test cells. Accordingly, the assay comprises the steps of first, contacting the test cell with a test compound for a time sufficient to inhibit the activity of a bacterial repressor ribosomal protein, and, next, measuring cell growth. An increase in cell growth in the presence of the test compound relative to the growth of the test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the repressor ribosomal protein.

The test cell for use in the fast-growth assay DNA containing a promoter and other regulatory sequences for expression of the repressor r-protein can be prepared by standard methods. The genes encoding the repressor r-proteins of *E. coli* and other bacterial species have been described in Section 5.1.1. If a fragment of the repressor r-protein is used, the fragment must be functionally capable of adversely affecting the growth of the test cell when expressed. For expression in the test cell, the promoter native to the repressor r-protein can be used as well as other promoters and regulatory sequences commonly used in the art for expression of proteins in the test cell. Promoter and other regulatory sequences may be isolated from an existing expression vector, generated by polymerase chain reaction or by chemical synthesis. A promoter can be operably linked to the 5' end of the coding region of a repressor r-protein gene by any known techniques. The choice of promoter and repressor r-protein fragment (if the use of a fragment is desirable) can be readily optimized by determining the growth rate of the combination in test cells containing the promoter and repressor r-protein fragment. The expression construct can exist in the test cell as a plasmid, a phage, or a cosmid. Alternatively, the expression construct may be integrated into and reside on the chromosome of the test cell.

For example, a family of plasmid vectors, namely the pET vectors (Novagen; see U.S. Patent No. 4,952,496 the disclosure of which is incorporated herein by reference), allow DNA fragments from virtually any source to be placed under control of the strong

promoter of ø10 for T7 RNA polymerase, or under control of this promoter plus the leader sequence of the T7 gene 10. These control sequences have been shown to be very efficient, and can easily be obtained and inserted in other plasmids, viruses or chromosomes to create a wide variety of other vectors and configurations for T7 RNA polymerase-directed expression of any repressor r-protein gene.

It is also useful if the amount of repressor r-protein present in a test cell can be regulated pursuant to the needs of the screening assay. For example, the promoter used in making the expression construct is an inducible promoter the transcriptional activity of which is responsive to the presence of an inducer molecule inside a test cell. Any inducible promoters known in the art, such as but not limited to, the lac promoter, can be used. Adjustment of the level of repressor r-protein in the cell can be accomplish through the addition of the lactose inducer, isopropyl-β-D-thiogalactopyranoside (IPTG).

5.3 Assays Based on Physical Binding

The methods of the present invention relate to *in vitro* screening assays for the identification of compounds that interfere with the binding of ribosomal proteins to RNA binding sites on a target RNA molecule. Such compounds may act as antagonists of ribosomal protein-RNA interactions in bacterial cells. Compounds identified by the binding assays described herein may therefore be novel antibiotic candidates, and can be analyzed further to test their effect on inhibiting bacterial cell growth in mammalian systems.

The ribosomal proteins that can be used in the present method comprise primary r-proteins and repressor r-proteins, as described in Sections 5.1.1 and 5.1.2, *supra*, or fragments thereof that comprise the RNA binding domain.

In the binding assays described herein, a bacterial r-protein is incubated with a target RNA molecule under conditions that allow RNA-protein interactions to occur, in the presence or the absence of a test compound. RNA-protein complexes are separated from the unbound RNA and/or protein component, and the complexes are then measured. A decrease in the formation of complexes in the presence of a test compound, relative to in its absence, identifies a candidate inhibitory compound.

Various methods may be used for separating RNA-protein complexes from the reaction. For example, in one embodiment, the RNA or protein component can be

attached to a solid phase surface, covalently or non-covalently. Such component can be attached to the support before the addition of the second component. The second component can then be passed over the support which now comprises the immobilized first component. Interaction between the two components thus occurs where one component is immobilized. In another embodiment, the binding interaction of the two components can take place in solution. The solution is then subsequently passed over a device or solid phase surface that separates the RNA-protein complexes from one or both of the two components.

Two methods for separating RNA-protein complexes, filter binding and affinity capture assays, are described in detail herein. The primary difference between these assays is the method used to separate the RNA-protein complexes from the unbound RNA and the unbound protein in each case. Briefly, a filter binding assay utilizes a device, usually a solid phase surface such as a filter or a column, to non-specifically retain proteins and/or protein-RNA complexes based on some physical or chemical difference between the complexes and the unbound reactants. On the other hand, an affinity capture assay, utilizes an immobilized binding partner to specifically capture the RNA or the protein in the binding reaction. The binding partner molecule of the affinity capture assay can comprise, for example, an antibody to the ribosomal protein, a complementary nucleic acid to the RNA, or a binding partner to an affinity tag present on either the RNA or the protein component of the mixture.

Chemical and enzymatic assays can be used to probe the state of RNA-protein interactions before and after the introduction of a novel compound. Such aasays based on modification protection and interference are described herein.

5.3.1 Filter-binding Assay

In one embodiment, a filter binding assay of the present invention can be used to separate and measure RNA bound to RNA-protein complexes in the presence of a test compound. In this assay, a ribosomal protein is mixed with a labeled RNA target molecule comprising an r-protein binding site, in the presence of a test compound, under conditions that allow RNA-protein complex formation. RNA-protein complexes and unbound protein are then separated from unbound target RNA. In a preferred embodiment, this is accomplished by passing the mixture over a device, such as a nitrocellulose filter,

that can retain proteins or r-protein/RNA complexes, but not free RNA. The labeled RNA bound to the protein retained on the solid phase surface can then be measured in the presence of the test compound, and in a control reaction without the test compound. Alternatively, the labeled target RNA molecule passing through the solid phase surface can be measured. The amount of RNA retained on the support, relative to either the input RNA or the RNA that passed through the filter, reflects the relative amount of RNA-protein complexes formed in each case.

The assay can be used to measure the effect of a test compound on the affinity of an r-protein for its binding site on an RNA molecule. A compound that interferes with the binding of an RNA to an r-protein will result in fewer labeled RNA molecules retained on the filter in the presence of the test compound than in its absence. Thus, if a lower level of bound RNA is measured in the presence of a test compound relative to its absence, a candidate inhibitor of r-protein-RNA interaction is identified.

In one embodiment, the r-protein is first titrated with target RNA to determine the appropriate concentration for performing competitive filter binding assays. To do this, r-protein is serially diluted and mixed with RNA under conditions that promote specific RNA-protein interactions. Either the r-protein or the target RNA can be labeled. The mixture of RNA and protein is then filtered through a device to separate the RNA-protein complexes from one of the components, either the RNA or the r-protein. The specific RNA-protein complexes are measured. A concentration is chosen equivalent to the minimum amount of protein required to specifically bind the maximal amount, i.e., saturate, the target RNA. The binding assay is repeated in the presence of test compounds at the optimal concentration of r-protein determined from the initial binding assay.

In a specific embodiment, r-protein is serially diluted in physiological buffer, such as 50 mM Tris/acetate, pH 7.5, 20 mM magnesium acetate, 270 mM KCl, and diluted to give a concentration range of 0.1 to 10000 nM. Labeled RNA is renatured at 42°C for 15 minutes, cooled to 4°C, added to the r-protein, and incubate on ice for approximately 30 minutes. This binding mixture is then filtered over nitrocellulose membranes, by methods well known in the art. Non-specific retention of RNA can be measured by filtering the reaction mixture in the absence of r-protein. An concentration of r-protein is selected that

gives a gives a specific retention of RNA in the range of approximately 30%. This concentration, in the range of 1-100 nM, is used to measure the effect of test compounds.

In another embodiment, ribosomal proteins can be adsorbed directly to a solid phase surface matrix such as, but not limited to, polycarbonate, polystyrene, polypropylene, polyethylene, glass, nitrocellulose, dextran, nylon, polyacrylamide and agarose. The support configuration can include beads, membranes, microparticles, the interior surface of a reaction vessel such as a microtiter plate, test tube or other reaction vessel.

In a specific embodiment, ribosomal protein is adsorbed to polycarbonate or glass beads by incubating proteins in a physiological buffer, such as PBS buffer, pH 6.9-7.7. Radioactively labeled target RNA is then added to the beads in the presence of compound. The effects of a test compound on the ability for r-protein to complex with mRNA is determined by measuring the level of radioactivity retained on the beads.

5.3.2 Affinity Capture Binding Assay

The affinity capture assay of the present invention utilizes a solid phase comprising a specific partner molecule of an affinity tag attached to either the protein or the target RNA component, or a specific partner molecule of the protein or target RNA component itself. The partner molecule can thus specifically capture, and remove the RNA or protein component from a binding reaction. This allows measurement of the binding affinity of an RNA and a protein, and allows comparison of relative binding affinities under different test conditions. Accordingly, a test compound that interferes with the interaction of a ribosomal protein and an RNA target molecule can be identified using this assay.

In one embodiment, where the solid phase comprises a partner molecule of an affinity tag or an r-protein itself, the r-protein is incubated with a labeled target RNA under conditions that allow r-protein-RNA binding to occur. The r-protein or the r-protein comprising the affinity tag can be attached to the solid phase surface prior to the binding reaction. Alternatively, the binding reaction can be contacted with the solid phase subsequent to the incubation. The labelled target RNA binds to the r-protein and is captured by the solid phase. The unbound RNA molecules are then removed from the solid phase and the amount of labeled RNA remaining associated with the solid phase is measured.

Alternatively, the amount of labeled target RNA that is not associated with the support can be measured. This binding reaction is performed in the presence and in the absence of a test compound. The amount of labeled target RNA bound to the r-protein is compared.

Alternatively, the solid phase can comprise a partner molecule of an RNA molecule. In this case, the affinity tagged target RNA molecule, comprising a binding site for the r-protein, is incubated with a labeled r-protein under conditions that allow binding to occur. The target RNA or the tagged target RNA can be pre-attached to the solid phase comprising a binding partner. Alternatively, the binding reaction can be contacted with the solid phase subsequent to the incubation. The labeled r-protein binds to the target RNA and is captured by the solid phase. The unbound proteins can then be separated from the solid phase and the labeled r-proteins bound to the solid phase or remaining in solution can then be measured. The binding reaction is performed in the presence and in the absence of a test compound.

5.3.3 Chemical and Enzymatic Modification Assays

Chemical and enzymatic modifications can be used to probe RNA secondary structure and RNA-protein interactions before and after the introduction of an antibiotic candidate compound. These assays are powerful tools that can be used for screening candidate drugs, understanding their mechanism of action, and designing novel drugs by site-specific modification of drug candidates.

Modifying enzymes and endonucleases are useful for probing RNA secondary structure and RNA– protein interactions. Non-limiting examples of such enzymes include RNase T1, S1 nuclease, which can be used to monitor unpaired nucleotides, and RNase V1, which can be used to probe base-paired or stacked nucleotides. Chemical probes can also be used to modify bases at accessible positions. Such chemical probes include, but are not limited to, DMS (dimethylsulfate, which methylates A residues at the N-1 position, C at the N-3 position and G at the N-7 position), DEPC (diethylpyrocarbonate, which ethylates A residues at the N-7 position), and CMCT (1-cyclohexyl-3(2-(1-methylmorpholino)-ethyl) carbodiimide-p-toluenesulfonate, which modifies U residues at N-3).

In one embodiment, an target RNA molecule is mixed with r-protein under conditions so as to allow binding to occur. In one reaction mixture, a test compound is added, while in another reaction mixture, the control, the test compound is omitted. An RNA modifying reagent is added to the binding reaction, and RNA-protein complexes are separated from the reaction. Any method known in the art can be used to separate RNA-protein complexes, such as the affinity capture assay and the filter binding assay described herein. In the case of chemical probing experiments, a reagent may be added to break the RNA strand at the modified position. Modifications are then detected by methods well known in the art, such as reverse transcription using a specific primer that flanks the RNA binding site (see, for eg., Mougel et al., 1988, Nucl. Acids Res. 16:28252839). RNA is then analyzed on a denaturing gel to reveal the modified or cleaved positions. The pattern of cleavage sites or modified bases from target RNA molecules bound in complexes formed in the presence of a compound is compared to RNA molecules bound in complexes formed in the absence of the compound.

The chemical and enzymatic probing assays described herein are powerful tools for analyzing candidate antibiotic molecules at the molecular level. They will be especially useful for analyzing the detailed mechanism of action of the drug candidate compounds that test positive in the screening assays of the invention. The understanding of such molecular detail will allow the introduction of alterations in a candidate compound to make even more potent forms of lead drug candidates. They will also be useful for screening antibiotic candidate compounds that are known to the disrupt ribosome assembly process.

5.3.4 Target RNA Molecules

A target RNA molecule useful in the present method comprises an RNA binding site for a specific r-protein. Such target RNA molecules include rRNAs, mRNAs, as well as analogs and fragments thereof, that contain binding sites for an r-protein.

In various embodiments, rRNAs, either 16S rRNA (GenBank Accession no. X80721; Cilia et al., 1996, Mol. Biol. Evol. 13:451-461), 23S rRNA (GenBank Accession no. X80721; Albrechtsen et al.,1991, Nucleic Acids Res. 19:1845-1852), or fragments thereof can be used as target RNAs for screening test compounds. In one embodiment, the

16S rRNA comprising the S8 recognition site is used, together with the S8 r-protein, in a binding assay. In a preferred embodiment, the RNA target molecule position comprising the central domain of the 16S rRNA molecule is used. Preferably, the central domain comprises RNA segments from about position 588 to about 606 and from about position 632 to about position 651 at the base of a hairpin (Gregory et al., 1984, J. Mol. Biol.178:287-302; Svensson et al., 1988, J. Mol. Biol. 200:301-308; Cerretti et al., 1988, J. Biol. Chem. 204:309; Gregory et al., 1988, 204:295-307). In another embodiment, the target RNA comprises residues from about positions 573 to about 865 of the 16S rRNA central domain.

In various embodiments, the polycistronic mRNA of an r-protein operon is used for screening for compounds that interfere with r-protein – RNA interactions. Such a polycistronic mRNA may encode, among other r-proteins, a repressor r-protein. As described above, the specific binding site for the repressor r-protein in several of the r-protein operons are known. Thus, it is not always necessary to use the entire polycistronic mRNA. In many cases, the repressor r-protein binding site on the mRNA is located in the leader sequence of a gene upstream of the gene that encodes the repressor r-protein. Some exemplary RNA binding sites for specific repressor r-proteins are provided herein.

In various preferred embodiments, the target RNA comprises a specific binding site for S8. In one specific preferred embodiment, a S8 RNA binding site analog is provided that comprises a hybrid of the mRNA and rRNA sequences, such that the secondary structure of the analog is functional equivalent and structurally similar to either the S8 mRNA binding site or S8 rRNA binding site, or both (see Figure 4). In another embodiment, the RNA binding site used in the binding assays is the mRNA binding site for S8 comprising the RNA segment between residues about +22 to about +81 of the *spc* operon (Gregory et al., 1988, J. Mol. Biol. 204:295-307). In another specific embodiment, the target RNA can comprise a larger segment of RNA from this region of the *spc* operon, comprising for example, residues +1 to about 150, +1 to about 300, or +1 to 1000 . Alternatively, the mRNA binding site for S8 can comprise the full-length polycistronic mRNA from the 5.9 Kb operon.

In another embodiment, the target RNA comprises an mRNA binding site for L4. In a specific embodiment, the mRNA binding site for L4 comprises the mRNA

transcript from the S10 operon, comprising the S10 leader sequence of the mRNA that contains the L4 binding site. In other specific embodiments, the mRNA binding site for L4 can comprise an RNA containing the sequence from nucleotide positions about +84 to about +192, +120 to +175, or about +1 to about +350, the regions that include the L4 binding site and sequences required for L4-mediated translational control.

In another embodiment, the RNA target molecule can comprise the mRNA transcribed from the alpha (α) operon. In a specific embodiment, the RNA comprises only the S4 binding site, from position about -84 to about +40 of the alpha mRNA. In another embodiment, the target RNA comprises the entire pseudoknot structure, from the +1 ribosome binding site to the proximal 40 bases of the S13 gene (Deckman & Draper, 1987, J. Mol. Biol. 196: 313-332) .

In another embodiment, the target RNA comprises an RNA transcribed from the *str* operon comprising the binding site of the S7 protein. In a specific embodiment, the target RNA comprises only the S7 binding site, the region between the genes for S12 and S7 (Post & Nomura, 1980, J. Biol. Chem. 255:4660-4666; GenBank Accession No. ES3149).

In yet another embodiment, the target RNA comprises an RNA transcribed from the S15 operon. S15 autogenously regulates its own synthesis. In a specific embodiment, the target RNA comprises the ribosome binding site from about +1 to about +100, comprising the S15 binding site (Philippe et al., 1990, J. Mol. Bio., 211:415; Portier et al., 1990, J. Mol. Bio., 211:407-414; Philippe et al., 1990, Proc. Natl. Acad. Sci. U.S.A., 90:4394).

In yet another embodiment, the target RNA comprises an RNA derived from the L11 operon, comprising the L1 binding site. In a specific embodiment, the RNA comprises from -52 to +9 of the L11 leader sequence (Kearney & Nomura, 1988, Mol. Gen. Genet., 210:60).

In yet another embodiment, the target RNA comprises an RNA derived from the *rif* operon. In a specific embodiment, the target RNA comprises positions -120 to -160 of the *rif* leader sequence. Alternatively, the target RNA can also include the double-hairpin loop structure that spans a region from about -212 to about -80 (Climie & Frieson, 1987, J. Mol. Biol, 198:371).

In yet another embodiment, the target RNA comprises an RNA derived from the L35 operon. In a specific embodiment, the target RNA comprises a region of the L35 leader sequence, and/or a region of the leader sequence of the *infC* gene (Lasagé et al., 1990, J. Mol. Biol., 213:465).

In yet another embodiment, the target RNA comprises an RNA derived from the S20 monocistronic operon. (Mackie, 1981, J. Biol. Chem., 256:8177-8182; GenBank Accession No. X04382). In yet another embodiment, the target RNA comprises an RNA derived from the S1 operon and may comprises an oligo(U)-containing sequences (Boni et al., 1991, Nuc. Acids Res., 19:155).

5.3.5 Construction of Ribosomal Protein Partner Molecules

An r-protein useful in the present method can be an r-protein comprising an affinity peptide tag. In various embodiments, such an affinity tag can be a peptide fused to the ribosomal protein. Such a fusion protein can be made by ligating an r-protein gene sequence to the sequence encoding the peptide tag in the proper reading frame. A variety of peptide tags known in the art may be used in the modification of an r-protein, such as but not limited to the polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan et al., 1987, Gene 67:21-30), and various cellulose binding domains (U.S. patent 5,496,934; 5,202,247; 5,137,819; Tomme et al., 1994, Protein Eng. 7:117-123), etc. Other peptide tags may impart fluorescent properties to an r-protein, e.g., portions of green fluorescent protein and the like. Other possible peptide tags are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other peptide tags are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid phase surface. The ribosomal protein gene product can be prepared using recombinant DNA techniques. For example, the ribosomal protein gene sequence can be introduced into a vector containing the sequence of a peptide tag, such that

the ribosomal gene is expressed as a peptide-tagged fusion protein. Peptide tags, which may be recognized by specific binding partners, may be used for affinity binding to the binding partner immobilized on a solid phase surface.

In one preferred embodiment, a poly-histidine tagged r-protein is constructed by insertion of a ribosomal protein gene or gene fragment comprising a ribosomal-binding site into an expression vector such as one of the pET15 series of vectors (Novagen), that express inserted sequences as fusion proteins with N-terminal poly-histidine tags. Proteins which have a succession of six or more histidine residues at their amino or carboxyl terminus have a strong binding affinity to nickel. Poly-histidine-tagged fusion proteins will bind specifically to the surface of a solid phase coated with chelated nickel. In a specific preferred embodiment, microtiter plates coated with metal chelates are used to capture the poly-histidine-tagged fusion proteins (Pierce). Labeled mRNA is then added to the plates in the presence of a test compound. The effects of the test compound on the ability for tagged r-protein to complex with the target RNA is determined by measuring the level of radioactivity retained on the plate. For example, a system described by Janknecht, *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88, 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+}-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

In another specific embodiment, an expression construct can be made by subcloning a ribosomal protein-binding site coding region sequence into the EcoRI restriction site of each of the three pGEX vectors (glutathione S-transferase expression vectors; Smith & Johnson, 1988, Gene 7:31-40). This allows for the expression of a fusion protein comprising the ribosomal protein linked to the GST binding domain in all three reading frames, such that, in one frame, the GST binding activity is maintained in the resulting fusion protein. A GST fusion peptide has a strong binding affinity for its substrate, glutathione. To assay for test compounds that interfere with ribosomal protein binding to a regulatory site on the ribosomal mRNA, resins linked to a binding partner or

compound can be used to bind affinity-labeled or fusion proteins. In a specific embodiment, r-proteins are separated from a binding mixture comprising a GST-fusion r-protein and radioactively labeled mRNA by contacting such mixture with a glutathione-linked solid phase surface, such as glutathione sepharose beads. For example, the GST-fusion protein can be anchored to glutathione-agarose beads or a glutathione-sepharose column. A labeled target RNA comprising the ribosomal protein binding site can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away. The interaction between the protein and the RNA can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A compound that interferes with the binding of an RNA to an r-protein will result in fewer labeled RNA molecules retained on the beads in the presence of the test compound than in its absence. Therefore, if a lower level of bound RNA is measured in the presence of a test compound relative to its absence, a candidate inhibitor of r-protein-RNA interaction is identified.

In another embodiment, a fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed.

In yet another embodiment, an affinity-tagged ribosomal protein can be constructed by conjugation of an affinity compound to the ribosomal protein. Affinity compounds can be used, such as, but not limited to, biotin, photobiotin, or other compounds known in the art. In one embodiment, affinity compounds or affinity tags can be conjugated to the ribosomal protein through a polyfunctional crosslinker, and preferably a bifunctional molecule. As used herein, the term polyfunctional crosslinker encompasses molecules having more than one functional group that reacts with a functional group on the ribosomal protein. Typically, such crosslinker forms covalent bonds with an amino or sulfhydryl group on a polypeptide. For example, biotin N-hydroxysuccinimide esters may be used.

5.3.6 Labeling of Ribosomal Proteins and Target RNA Molecules

The target RNA molecule used in the physical binding assays described herein, comprises an RNA binding site for a ribosomal protein. Such an RNA binding site can be rRNA or an analog or fragment thereof. In addition, where the ribosomal protein is

also a translational repressor protein, the RNA binding site can be an mRNA, or a fragment thereof. Such molecules can be prepared by a variety of well-known methods (Ausubel, *supra*). For example, in one embodiment, an RNA molecule can be isolated as a natural product of cells, such as a 16S or 23S rRNA, or a fragment thereof. In another embodiment, RNA can be prepared by recombinant DNA methods, such as cloning a rRNA gene sequence or r-protein gene sequence in a vector downstream of a RNA polymerase promoter. The nucleotide sequences of non-limiting examples of ribosomal RNA genes that can be expressed by methods of the invention are published as follows: 16S rRNA, GenBank Accession No. X0721 (Cilia et al., 1996, Mol. Biol. Evol. 13:451-461). Vectors that can be used to express RNA, containing T3, T7, or SP6 RNA polymerase promoters, are well known in the art (Ausubel, *supra*).

Target RNA molecules can be labeled by direct or indirect labeling methods known in the art using an isotopic or non-isotopic label. The "label" refers to a moiety, such as a radioactive isotope or group containing same, and nonisotopic labels, such as enzymes, biotin, avidin, streptavidin, digoxygenin, luminescent agents, dyes, haptens, and the like. Luminescent agents, depending upon the source of exciting energy, can be classified as radioluminescent, chemiluminescent, bioluminescent, and photoluminescent (including fluorescent and phosphorescent). In a preferred embodiment the detectable marker is biotin.

In one embodiment, the target RNA is labeled directly by methods known in the art. Such methods include direct chemical modification or enzymatic labeling of the target RNA. For example, target RNA molecules can be labeled using polynucleotide kinase and a labeled NTP. In another embodiment, the target RNA is labeled synthetically, for example, by *in-vitro* transcription of a DNA sequence encoding the target RNA gene in the presence of a labeled nucleotide triphosphate such as, but not limited to, ^{32}P-NTP, ^{33}P-NTP, ^{35}S-NTP, ^{15}C-NTP, and ^{13}C-NTP. Labeled RNA is then detected by such means as the use of a gamma counter, a scintillation counter, or autoradiography. In another embodiment, RNA is labeled by incorporation of hapten-labeled nucleotides such as digoxigenin-labeled nucleotides (Holtke & Kessler 1990), and the hapten-labeled RNA is detected using an antibody that specifically binds to the hapten, for example anti-DIG-alkaline phosphatase or POD conjugate (Fab fragments). Such compounds can be detected

using chemiluminescent, colorimetric, or fluorescent substrates. In yet another embodiment, RNA can be labeled with biotinylated nucleotides, and detected using a streptavidin-linked detectable compound.

Ribosomal proteins may be labeled with a detectable marker, using methods for protein labeling known in the art. A "detectable marker" refers to a moiety, such as a radioactive isotope or group containing same, or nonisotopic labels, such as enzymes, biotin, avidin, streptavidin, digoxygenin, luminescent agents, dyes, haptens, and the like. Luminescent agents, depending upon the source of exciting energy, can be classified as radioluminescent, chemiluminescent, bioluminescent, and photoluminescent (including fluorescent and phosphorescent).

In one embodiment of the invention, the affinity capture assay may be performed using a scintillation proximity assay (SPA, Amersham). Either the RNA component or the r-protein component can be labeled with a biotinylated marker, and the other component can be labeled with a radioactive label such as ^{33}P. For example, an labeled RNA can be generated by standard *in vitro* transcription with the inclusion of a ^{33}P-labeled nucleotide triphosphate, for example, ^{33}P-CTP. The two components can be mixed together, under conditions that allow RNA -protein interactions to occur. The biotinylated r-protein and the labelled RNA-r-protein complexes can then be captured on streptavidin beads, and counted in a scintillation counter.

5.4 Structure – Function Analysis

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can inhibit ribosome assembly or disrupt ribosomal protein synthesis. Having identified such a compound or composition, the active sites or regions of a ribosomal protein or an RNA binding site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of RNA, or from study of complexes of the test compound with ribosomal protein and/or RNA. In certain case, chemical or X-ray crystallographic methods can be used to find the binding site by finding where on the ribosomal protein and/or RNA the bound compound is found.

Next, the three dimensional geometric structure of the binding site of the ribosomal protein and/or RNA is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site, either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential compounds that blocks the function of the ribosomal protein of interest.

Alternatively, these methods can be used to identify improved inhibitory compounds from an already known inhibitory compound. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered structure is then compared to the active site structure of the

compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain inhibitory compounds of improved specificity or activity.

Further experimental and computer modeling methods useful to identify compounds based upon identification of the binding sites on ribosomal protein, and RNA, and related translation factors will be apparent to those of skill in the art. Of the approximately 52 r-proteins of *E. coli*, high resolution structures of nine have been determined, six from the large subunit. L7/L12, L6, L9, L14 and L1 (Leijonmarck & Liljas 1987, J. Mol. Biol. 195:555-580; Wilson et al., 1986, Proc. Natl. Acad. Sci. USA 83:7251-7255; Golden at al. 1993, EMBO J. 12:4901-4908; Hoffman et al. 1994, EMBO J. 13:205-212; Davies et al. 1996, Structure 4:55-66; Nikonov et al. 1996, EMBO J. 15:1350-1359), and three from the small subunit: S5, S6, S17 and S8 (Ramakrishnan & White 1992, Biochem. and Cell Biol. 73:979-986; Lindahl et al. 1994, EMBO J. 13:1249-1254; Jaishree et al., 1996, Biochemistry 35:2845-2853; Davies et al., 1996, Structure 9:1093-1104). The structural information on these ribosomal proteins can readily be adapted for purposes of modeling the binding of a test compound, and further database searching.

Examples of molecular modeling systems are the CHARMm and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen et al. (1988, Acta Pharmaceutical Fennica 97:159-166); Ripka (1988 New Scientist 54-57); McKinaly & Rossmann (1989, Annu. Rev. Pharmacol. Toxiciol. 29:111-122); Perry & Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 Alan R. Liss, Inc. 1989; Lewis & Dean (1989, Proc. R. Soc. Lond. 236:125-140 and 141-162); and, with respect to a model receptor for nucleic acid components, Askew, et al. (1989, J. Am. Chem. Soc. 111:1082-1090). Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario,

Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

5.5 Antibiotic Agents Identified by the Assays

In yet another embodiment, the invention provides novel antibiotic agents discovered by the methods described above. These antibiotic agents are capable of disrupting ribosomal protein biosynthesis and ribosome assembly. These agents may, for example, act by inhibiting the binding of repressor r-protein to its binding site on mRNA and/or inhibit the binding of primary binding r-protein to rRNA, and are expected to be effective in a variety of species of bacteria, including infectious pathogenic bacteria. The invention also includes novel pharmaceutical compositions which comprise antibiotic agents discovered as described above formulated in pharmaceutically acceptable formulations.

In another embodiment, the invention features a method for treating a subject infected with an infectious agent by administering to that subject a therapeutically effective amount of an antibiotic agent which disrupts ribosomal protein biosynthesis and/or ribosome assembly in the infectious agent as determined by the assays of the invention. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

In yet another embodiment, antibiotic agents of the present invention can be used to treat contaminated items, such as crops, wood, metal or plastic and the like, by methods such as, but not limited to, spraying or dusting of that agent onto the contaminated item, or impregnating that agent into the item.

By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either

partially or completely, physiological or biochemical parameters associated with or causative of a bacterial disease or condition.

5.5.1 Determination of MIC

The minimum inhibitory concentration (MIC) against bacterial organisms is determined for each test compound that is positive in the assay. Methods known in the art may be used such as broth microdilution testing, using a range of concentrations of each test compound (1993, National Committee for Clinical Laboratory Standards). Methods for Dilution Antimicrobial Susceptibility Tests For Bacteria That Grow Aerobically - Third Edition: Approved Standard, M7-A3). The MIC against a variety of pathogens are determined using the same method. Pathogenic species to be tested generally include: *E. coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, *Shigella flexneri*, and *Salmonella typhimurium*.

5.5.2 Cytotoxicity Testing

Unfortunately, toxicity does not always arise from the same mechanism of action as is responsible for growth inhibition in the targeted microorganism. Therefore, the selectivity of the target should not be assessed solely on the basis of these results.

Cytotoxicity can be measured by methods known in the art. One such method is assessing growth of mammalian cells in the presence of the test compound, using a protein binding dye, sulforhodamine B (SRB). SRB binds electrostatically to basic amino acids. Binding and solubilization of the dye can be controlled by changes in pH. SRB binds stoichiometrically to proteins in one pH range but can be solubilized and extracted for measurement in another. An increase in total protein is correlated to cell growth. Cell growth in the presence of compound is compared to growth without added compound to establish a growth inhibitory concentration (GI_{50}) (Skehan et al., 1990, J. Natl. Cancer. Inst., 82:1107-1112). Another method of measuring cytoxicity which may be used in an assay containing 3[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide/2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt ("MTT/XTT") as

described in Mosmann T., 1983, J. Immunol. Methods, 65:55-63, which is incorporated by reference in its entirety for all purposes.

5.5.3 Formulation

The antibiotic compounds identified by methods of the invention may be formulated into pharmaceutical preparations for administration to animals for treatment of a variety of infectious diseases. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may be prepared, packaged, labeled for treatment of and used for the treatment of the indicated infectious diseases caused by microorganisms, such as those listed *infra*.

If the antibiotic compound is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, polyethylene glycol or glycerine. Thus, the compounds and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, topical, dermal, vaginal, rectal administration and drug delivery device, *e.g.*, porous or viscous material, such as lipofoam.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or

silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The antibiotic compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the antibiotic compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the antibiotic compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a

sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The antibiotic compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The pharmaceutical compositions of the present invention comprise an antibiotic compound as the active ingredient, or a pharmaceutically acceptable salt thereof, and may also contain a pharmaceutically acceptable carrier, and optionally, other therapeutic ingredients, for example antivirals. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic acids and bases, including inorganic and organic acids and bases.

The pharmaceutical compositions include compositions suitable for oral, rectal, mucosal routes, transdermal, parenteral (including subcutaneous, intramuscular, intrathecal and intravenous), although the most suitable route in any given case will depend on the nature and severity of the condition being treated.

In practical use, an antibiotic agent can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including tablets, capsules, powders, intravenous injections or infusions). In preparing the compositions for oral dosage form any of the usual pharmaceutical media may be employed, e.g., water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, and the like; in the case of oral liquid preparations, e.g., suspensions, solutions, elixirs, liposomes and aerosols; starches, sugars, micro-crystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like in the case of oral solid preparations e.g., powders, capsules, and tablets. In preparing the compositions for parenteral dosage form, such as intravenous injection or infusion, similar pharmaceutical media may be employed, e.g., water, glycols, oils, buffers, sugar, preservatives and the like know to those skilled in the art. Examples of such parenteral compositions include, but are not limited to Dextrose 5%w/v, normal saline or other solutions.

5.5.4 Administration

For administration to subjects, antibiotic compounds discovered by using the assays of the invention are formulated in pharmaceutically acceptable compositions. The compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonially, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, or intraperitoneally.

As will be readily apparent to one skilled in the art, the magnitude of a therapeutic dose of an antibiotic compound in the acute or chronic management of an infectious disease will vary with the severity of the condition to be treated, the particular composition employed, and the route of administration. The dose, and perhaps dose frequency, will also vary according to the species of the animal, the age, body weight, condition and response of the individual subject. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

Desirable blood levels may be maintained by a continuous infusion of an antibiotic compound as ascertained by plasma levels. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

Optionally, a second antibacterial compound may be used in combination with the compound identified by the method of the invention. The second antibacterial compound may be naturally occurring or synthetic. Suitable naturally occurring antibacterial compounds include, but are not limited to, aminoglycosides (including but not limited to dihydrostreptomycin, gentamycin, kanamycin, neomycin, paromycin and streptomycin); amphenicols (including but not limited to chloramphenicol); ansamycins (including but not limited to rifamycin); β-lactams such as carbapems (including but not limited to imipenem), cephalosporins (including but not limited to cefazedone and

cefroxadine), cephamycins (including but not limited to cefbuperazone); monobactams (including but not limited to aztreonam), oxacephems (including but not limited to flomoxef) or penicillins (including but not limited to ampicillin, carbencillin, methicillin, penicillin N, penicillin O and penicillin V); lincosamides (including but not limited to clindamycin and lincomycin); macrolides (including but not limited to carbomycin and erythromycin); polypeptides (including but not limited to gramicidin S and vancomycin); tetracyclines (including but not limited to apicycline, methacycline and tetracycline); and others such as cycloserine, mupirocin and tuberin. Suitable synthetic antibacterial compounds include 2,4-diaminopyrimidines (including but not limited to trimethoprim); nitrofurans (including but not limited to nifuradene); quinolones and quinolone analogs (including but not limited to enoxacin, lomefloxacin, nalidixic acid and ofloxacin); sulfonamides (including but not limited to sulfamoxole and sulfanilamide); sulfones (including but not limited to diathymosulfone); oxazolidinones (including but not limited to linezolid); and others such as glycylcycines, clofoctol, hexedine, methenamine, and nitroxoline.

The "adjunct administration" of a compound identified by the method of the invention and a second antibacterial compound means that the two are administered either as a mixture or sequentially. When administered sequentially, the compound may be administered before or after the second antibacterial compound, so long as the initially administered compound is still providing antibacterial activity. Any of the above described modes of administration can be used in combination to deliver the compound and the second antibacterial compound. When a compound identified by the method of the invention and a second antibacterial compound are administered adjunctively as a mixture, they are preferably given in the form of a pharmaceutical composition comprising both agents. Thus, in a further embodiment of the invention, it is provided a pharmaceutical composition comprising a compound identified by the method of the invention and a second antibacterial compound together with a pharmaceutically acceptable carrier.

In selected cases, drug delivery vehicles may be employed for systemic or topical administration. They can be designated to serve as a slow release reservoir, or to deliver their contents directly to the target cell. Such vehicles have been shown to also increase the circulation half-life of drugs which would otherwise be rapidly cleared from the

blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, and bioadhesive microspheres. These vehicles have been developed for chemotherapeutic agents.

Topical administration of agents is advantageous when localized concentration at the site of administration with minimal systemic adsorption is desired. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be administered is far less than that required for other administration routes.

Antibiotic agents may also be systemically administered. Systemic absorption refers to the accumulation of agents in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: oral, intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ocular. Each of these administration routes exposes the agent to an accessible target.

5.6 Target Infectious Agents

The antibiotic compounds identified by the methods of the infection can be used to treat infectious diseases in animals, including humans, companion animals (*e.g.*, dogs and cats), livestock animals (e.g., sheep, cattle, goats, pigs, and horses), laboratory animals (*e.g.*, mice, rats, and rabbits), and captive or wild animals.

Specifically, infectious diseases caused by bacteria including but not limited to, gram positive cocci, such as Staphylococci (e.g., *S. aureus*), Streptococci (e.g., *S. pneumoniae, S. pyrogens, S. faecalis, S. viridans*); gram positive bacilli, such as Bacillus (e.g., *B. anthracis*), Corynebacterium (e.g., *C. diphtheriae*), Listeria (e.g., *L. monocytogenes*); gram negative cocci, such as Neisseria (e.g., *N. gonorrhoeae, N. Meningitidis*); gram negative bacilli, such as Haemophilus (e.g. *H. influenzae*), Pasteurella (e.g., *P. multocida*), Proteus (e.g., *P. mirabilis*), Salmonella (e.g., *S. typhi murium*), Shigella species, Escherichia (e.g., *E. coli*), Klebsiella (e.g., *K. pneumoniae*), Serratia (e.g. *S. marcescens*), Yersinia (e.g., *Y. pestis*), Providencia species, Enterobacter species, Bacteroides (e.g., *fragilis*), Acinetobacter species, Campylobacter (e.g., *C. jejuni*), Pseudomonas (e.g., *P. aeruginosa*), Bordetella (e.g., *B. pertussis*), Brucella species, Fracisella (e.g., *F. tularensis*), Clostridia (e.g., *C. perfriugens*), Helicobacter (e.g., *H.*

pylori), Vibrio (e.g., *V. cholerae*), Mycoplasma (e.g., *M. pneumoniae*), Legionella (e.g., *L. pneumophila*), Spirochetes (e.g. *Treponema, Leptospira* and *Borrelia*), Mycobacteria (e.g., *M. tuberculosis*), Nocardia (e.g., *N. asteroides*), Chlamydia (e.g., *C. trachomatis*), and Rickettsia species, can be treated by antibiotic drugs discovered by the methods of the invention.

6. EXAMPLE

Presented herein is an example of the design and construction of an RNA target molecule and of its use in the translation assay of the invention. The target RNA molecule comprises an RNA binding site analog that specifically interacts with the primary binding and repressor protein S8.

6.1 Design of the RNA Binding Site and Reporter Construct

An optimal S8 RNA binding site was modeled by comparing the sequence and secondary structures of S8 mRNA and rRNA binding sites in *E. coli*. The S8 protein binds to rRNA at approximately 588-606 and 632-651 at the base of a stem-loop-stem hairpin located in the central domain of 16S rRNA (Svensson et al., 1988; Cerretti et al., 1988; and Gregory et al., 1988). The nucleotide sequence requirements for binding of S8 to this region of RNA have been studied in detail (Wu et al., 1994, Nucleic Acids Res. 22:1687-95). These studies indicated that the minimal structural requirements in the 16S rRNA included a stem-loop-stem structure. The bulged nucleotides in the stem of the hairpin of the mRNA are known to result in a lower affinity for the S8 protein.

A RNA binding site analog was constructed for use in the translation assays. The analog was designed to more closely mimic the rRNA binding site, which binds more efficiently than the mRNA binding site. The sequence of the resulting analog is shown in Figure 4. The bulged RNA pairs in the base of the stem were eliminated, and an AUG start codon was inserted to allow translation initiation of the reporter RNA.

To construct a reporter gene, the control region was designed based on the architecture of the native *spc* polycistronic RNA which is shown in Figure 5. The reporter gene, Renilla luciferase, was placed under the control of an S8 RNA binding site constructed as described above. The final construct is shown in Figure 6. In addition, upstream of the luciferase gene, a strong ribosome binding site, or Shine Delgarno (SD)

sequence, an initiator AUG, and a mini orf (open reading frame) was positioned to mimic the native position of the S8 binding site in the *spc* polycistronic mRNA. The orf, mimicking the ribosomal protein, L5, was located upstream of S8 in the *spc* operon, followed by the S8 translational control region. A 14-base pair spacer containing a second strong SD sequence separated the two reading frames. The intervening RNA between the RNA that forms the recognition stem (the loop-stem of the stem-loop-stem structure) was truncated to a minimal UUUUU sequence, See Figure 6B. The reporter gene was constructed by standard techniques of molecular cloning.

6.2 *In Vitro* Translation Assays

The results of *in vitro* translation assays using a reporter construct that encodes an RNA binding site analog is shown in Figure 7. Recombinant S8 His-tagged S8 protein (0, 0.5, 1, 2, 3, or 4 micrograms) were added to *in vitro* translation extracts. After an initial increase in Renilla luciferase activity, Renilla luciferase activity becomes suppressed with increasing S8 concentration. The initial increase in activity may be due to a boost in translation efficiency caused by the addition of the S8 r-protein. However, after this initial increase, the sharp decline indicates that translation is being efficiently repressed by S8 binding at the S8 RNA binding site. Any test compound added to the translation reaction which restores or increase Renilla luciferase activity is a candidate for further studies.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1. A method for screening for a test compound comprising :

a) contacting a test cell with a test compound for a time period sufficient to allow the test compound to inhibit the activity of a bacterial ribosomal protein, wherein the test cell contains a reporter mRNA that comprises an RNA binding site of the ribosomal protein such that translation of the reporter is repressed by the ribosomal protein; and

b) measuring the translation of the reporter mRNA, wherein a specific increase in the translation of the reporter mRNA in the test cell contacted with the test compound relative to the translation of the reporter mRNA in a test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein in the test cell.

2. A method for screening for a test compound comprising:

a) culturing a test cell under selection; wherein the test cell contains a nucleic acid sequence encoding a bacterial ribosomal protein operably linked to a promoter such that the ribosomal protein is expressed in the test cell;

b) contacting the test cell with a test compound for a time sufficient to inhibit the activity of the ribosomal protein; and

c) measuring the growth of the test cell, wherein an increase in growth of the test cell contacted with the test compound relative to the growth of the test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein.

3. The method of claim 1 wherein the reporter mRNA is produced in the test cell by a reporter gene.

4. The method of claim 1 or 2, wherein the test cell is an *E. coli* cell.

5. The method of claim 1 or 2, wherein the ribosomal protein is S8.

6. The method of claim 1 or 2, wherein the ribosomal protein is S1, S4, S7, S15, S20, L1, L4, L10 or L20.

7. The method of claim 1 or 2, wherein the reporter gene is chloramphenicol acetyl transferase, luciferase, green fluorescent protein, β-galactosidase, β-lactamase, or β-glucuronidase.

8. A method for screening for a test compound comprising:

a) contacting a test compound with a reaction mixture comprising a bacterial ribosomal protein and a reporter mRNA for a time period sufficient to allow the test compound to inhibit the activity of the bacterial ribosomal protein, wherein the reporter mRNA comprises the RNA binding site of the ribosomal protein such that translation of the reporter mRNA is repressed by the ribosomal protein; and

b) measuring the translation of the reporter mRNA, wherein a specific increase in the translation of the reporter mRNA in the test cell contacted with the test compound relative to the translation of the reporter mRNA in a test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein.

9. The method of claim 8 wherein the reaction mixture in step (a) comprises an excess of a recombinant ribosomal protein.

10. The method of claim 1, 2, 3, or 8 wherein the translation of the reporter mRNA is detected by a signal generated by the reporter.

11. A method for screening for a test compound comprising:

a) contacting a bacterial ribosomal protein with an RNA molecule comprising a binding site specific for the ribosomal protein in the presence of a test compound for a time period sufficient to allow binding of the ribosomal protein to the RNA;

b) detecting the binding of the ribosomal protein to the RNA, wherein a decrease in the binding of the ribosomal protein to the RNA contacted with the test compound relative to the binding of the ribosomal protein to the RNA not contacted with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA.

12. A method for screening for a test compound that inhibits bacterial growth comprising:

a) contacting a bacterial ribosomal protein comprising an affinity tag with an RNA molecule comprising a binding site specific for the ribosomal protein, in the presence of a test compound for a time period sufficient to allow binding of the ribosomal protein to the RNA;

b) contacting the ribosomal protein with a solid phase surface containing a binding partner of the affinity tag, for a time period sufficient to allow binding of the affinity tag on the ribosomal protein to the binding partner on the solid phase;

c) removing the RNA molecules that are not bound to the ribosomal protein; and

d) detecting the binding of the ribosomal protein to the RNA, wherein a decrease in the binding of the ribosomal protein to the RNA contacted with the test compound relative to the binding of the ribosomal protein to the RNA not contacted

with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA.

13. A method for screening for a test compound comprising:

a) contacting a bacterial ribosomal protein with an RNA molecule comprising a binding site for the ribosomal protein and an affinity tag, in the presence of a test compound for a time period sufficient to allow binding of the ribosomal protein to the RNA;

b) contacting the RNA with a solid phase containing a binding partner of the affinity tag, for a time period sufficient to allow binding of the affinity tag on the RNA to the binding partner on the solid phase;

c) removing the ribosomal protein that are not bound to the RNA; and

d) detecting the binding of the ribosomal protein to the RNA, wherein a decrease in the binding of the ribosomal protein to the RNA contacted with the test compound relative to the binding of the ribosomal protein to the RNA not contacted with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA.

14. The method of claim 11, 12, or 13, wherein the ribosomal protein is S8.

15. The method of claim 11, 12, or 13, wherein the ribosomal protein is S4, S7, S8, S15, S17, S20, L1, L2, L3, L4, L7/12, L10, L11, L15, L20, L23 or L24.

16. A method for inhibiting the growth of bacteria comprising contacting the bacteria with a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, 2, or 3.

17. A method for inhibiting the growth of bacteria comprising contacting the bacteria with a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13.

18. A compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, or 2.

19. A compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13.

20. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, or 2.

21. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13.

22. A method for treating a mammal with an infectious disease caused by bacteria comprising administering to the mammal a therapeutically effective amount of a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, or 2.

23. A method for treating a mammal with an infectious disease caused by bacteria comprising administering to the mammal a therapeutically effective amount of a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13.

24. The method of claim 22 wherein the mammal is human.

25. The method of claim 23 wherein the mammal is human.

26. The method of claim 22 further comprising adjunctively administering to the mammal a second antibacterial compound.

27. The method of claim 26 wherein the second antibacterial compound is a member of an antibiotic group selected from the group consisting of aminoglycosides, amphenicols, ansamycins, β-lactams, cephalosporins, cephamycins, monobactams, oxacephems, penicillins, lincosamides, macrolides, polypeptide antibiotics, tetracyclines, 2,4-diaminopyrimidines, nitrofurans, quinolones, sulfonamides, sulfones, oxazolidinones and glycylcycines.

28. The method of claim 23 further comprising adjunctively administering to the mammal a second antibacterial compound.

29. The method of claim 28 wherein the second antibacterial compound is a member of an antibiotic group selected from the group consisting of aminoglycosides, amphenicols, ansamycins, β-lactams, cephalosporins, cephamycins, monobactams, oxacephems, penicillins, lincosamides, macrolides, polypeptide antibiotics, tetracyclines, 2,4-diaminopyrimidines, nitrofurans, quinolones, sulfonamides, sulfones, oxazolidinones and glycylcycines.

30. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, or 2, a second antibacterial compound, and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13, a second antibacterial compound, and a pharmaceutically acceptable carrier.

spc operon	L14	L24**L5	S14	S8	L6	L18	S5	L30	L15	secY	L36	
S10 operon	**S10	L3	L4	L23	L2	S19	L22	S3	L16	L29	S17	
a operon	**S13	S11	S4	a	L17							
str operon	S12	**S7	EF-G	EF-Tu								
L11 operon	**L11	L1										
L10 operon	**L10	L7/12	att	b	b'							
S15 operon	**S15	ter	pnp									
L35 operon	IF3	**L35	L20									
S20 operon	S20											
S1 operon	S1											

FIG. 1

```
                1       10        20        30        40        50        60
                |-------+---------+---------+---------+---------+---------+---------
E.coli          MSMQDPIADMLTRIRNGQAANKAAVTMPSSKLKVAIANVLKEEGFIEDFKVEGDTKP--E-LELTLKYF
H.influenzae    MSMQDPIADMLTRIRNGQAANKVAINMPSSKLKVAIANVLAAEGYIESVKVLEGAKP--E-LEITLKYF
M.tuberculosis  MTMTDPIADFLTRLRNANSAYHDEVSLPHSKLKANIAQILKNEGYISDFRTEDARVG--KSLVIQLKYG
M.leprae        MTMTDPIADFLTRLRNANSAYHDEVTVPHSNIKANIAQILKNEGYIRDFRTEDARVG--KSLIIQLKYG
B.subtilis      MVMTDPIADMLTRIRNANMVRHEKLEIPASKLKREIAEILKREGFIRDVEFVEDSKQ--GIIRVFLKYG
M.pneumoniae        DPVADLLTKINNARKAKLMTVTTIASKLKIAILEILVKEGYLANFQVLENKSKTKRIVTFNLKYT
M.genitalium        DPVSDLFTKINNARKAKLLTVTTIASKLKIAILEILIKEGYLANYQVLENKTKTKKLVSFTLKYT
C.trachomatis       DSIANLLTRIRNALMAEHLYIDIEHSKMLEAIVRILKQHGFIAHFLVKEENRK--RLMRVFLRYG
H.pylori          VNDIIADSLTRLRNASMRRLEFTQLYYAKIVVSILEIFKEKGFIKDFNVKDKDKK--QSVYVQLAYD
Consensus           Dp!A# LtrirNa  a         sKlk aI e!lk eG%i df v #   k        LkY

                70        80        90        100       110       120       130 134
                +---------+---------+---------+---------+---------+---------+---|
E.coli          QGKA-VVESIQRVSRPGLRIYKRKDQLPKVMAGLGIAVVSTSKGVMTDRAARQAGLGGEIICYVA
H.influenzae    QGKP-VVESIQRVSRPGLRIYKRKDELPKVMGGLGVAVISTSKGVMTDRAARQAGLGGEIICYVA
M.tuberculosis  PSRERSIAGLRRVSKPGLRVYAKSTNLPRVLGGLGVAIISTSSGLLTDRQAARQGVGGEVLAYV
M.leprae        PSRERSIAGLRRVSKPGLRVYAKSINLPRVLGGLGVVIISTSSGLLTDRQAARQGVGGEVLAYV
B.subtilis      QNNERVITGLKRISKPGLRVYAKSNEVPRVLNGLGIAIISTSQGVLTDKEARAKQAGGEVLAYV
M.pneumoniae    QRRIPSINGVKQISKPGLRIYRPFEKLPLVLNGLGIAIISTSDGVMTDKVARLKKIGGEILAYV
M.genitalium    QRRICSINGVKQISKPGLRIYRSFEKLPLVLNGLGIAIISTSDGVMTDKVARLKKIGGEILAYV
C.trachomatis   EDRRPVIHALKRVSKPSRRVYVSAAKIPYVFGNMGIAVLSTPQGVLEGSVARAKNVGGELLCLV
H.pylori        EKGHSKISEVKRLSKPGRRVYKQKNELKRFKNGYGVIVVSTSKGVITNEEAYRQNVGGEVLCSI
Consensus       q r   !   krvSkPglR!Y     lp v nglG!a!iSTs Gv td  Ar    GGE lcy!
```

FIG.2

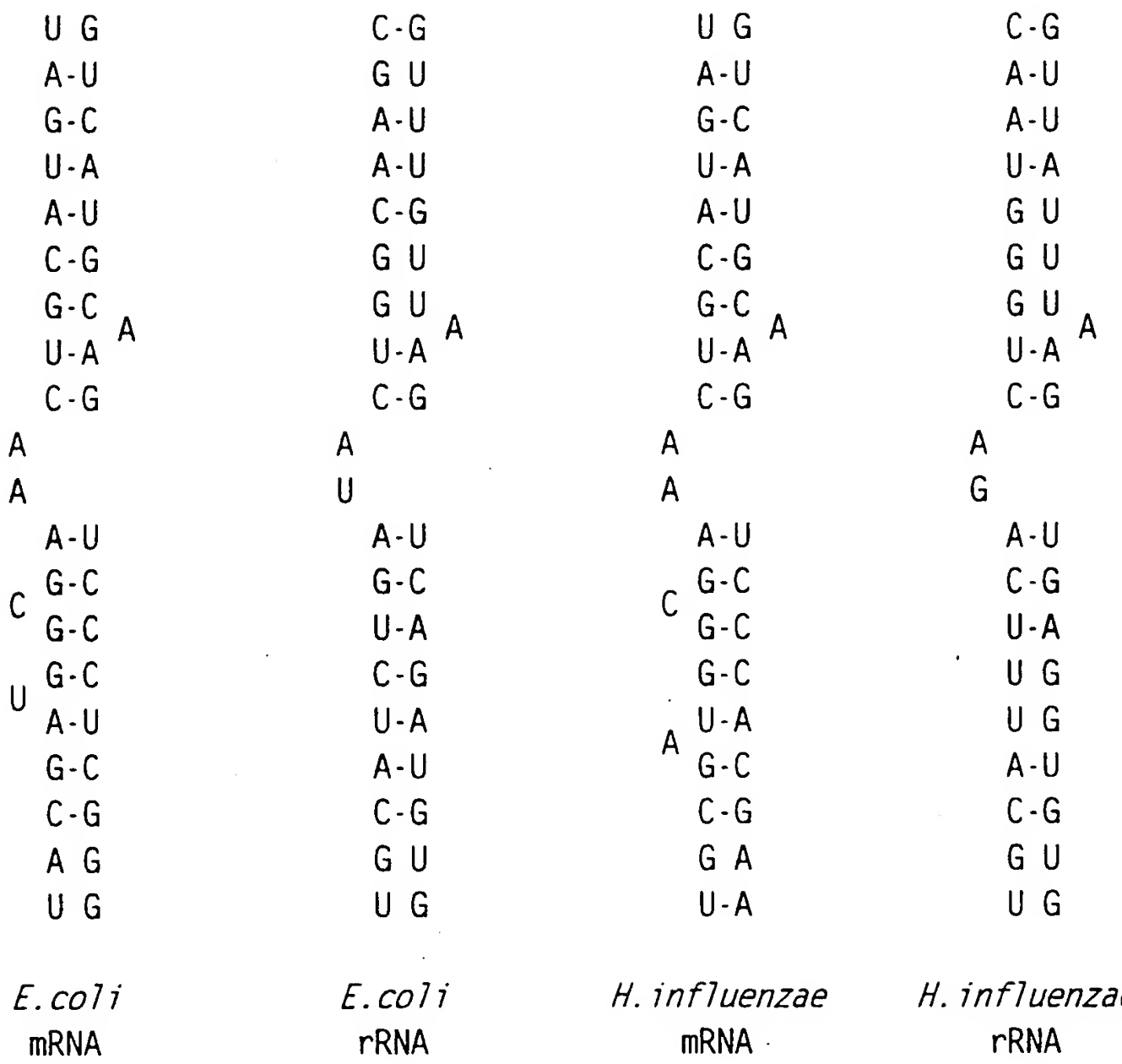
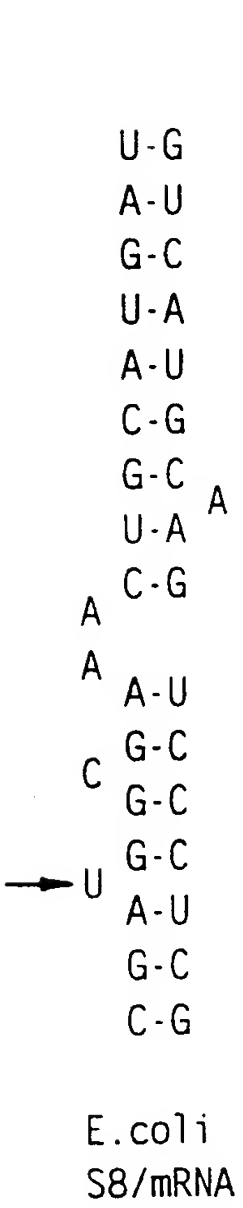
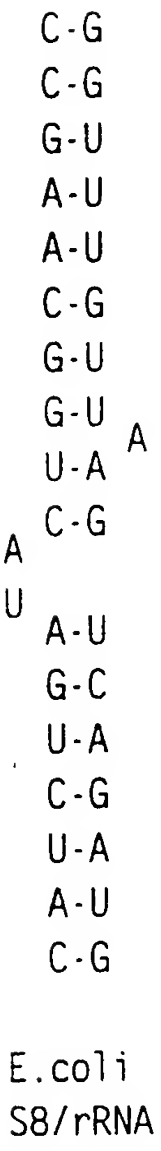


FIG.3





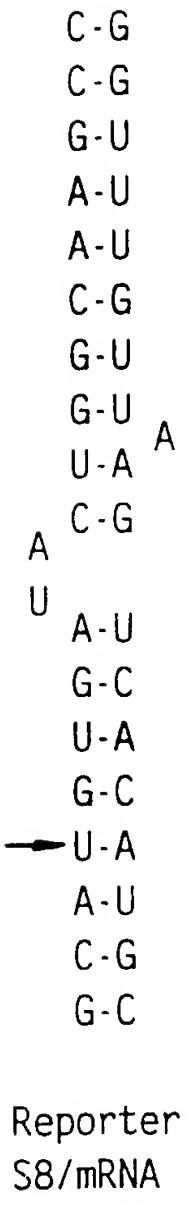


FIG.4A FIG.4B FIG.4C

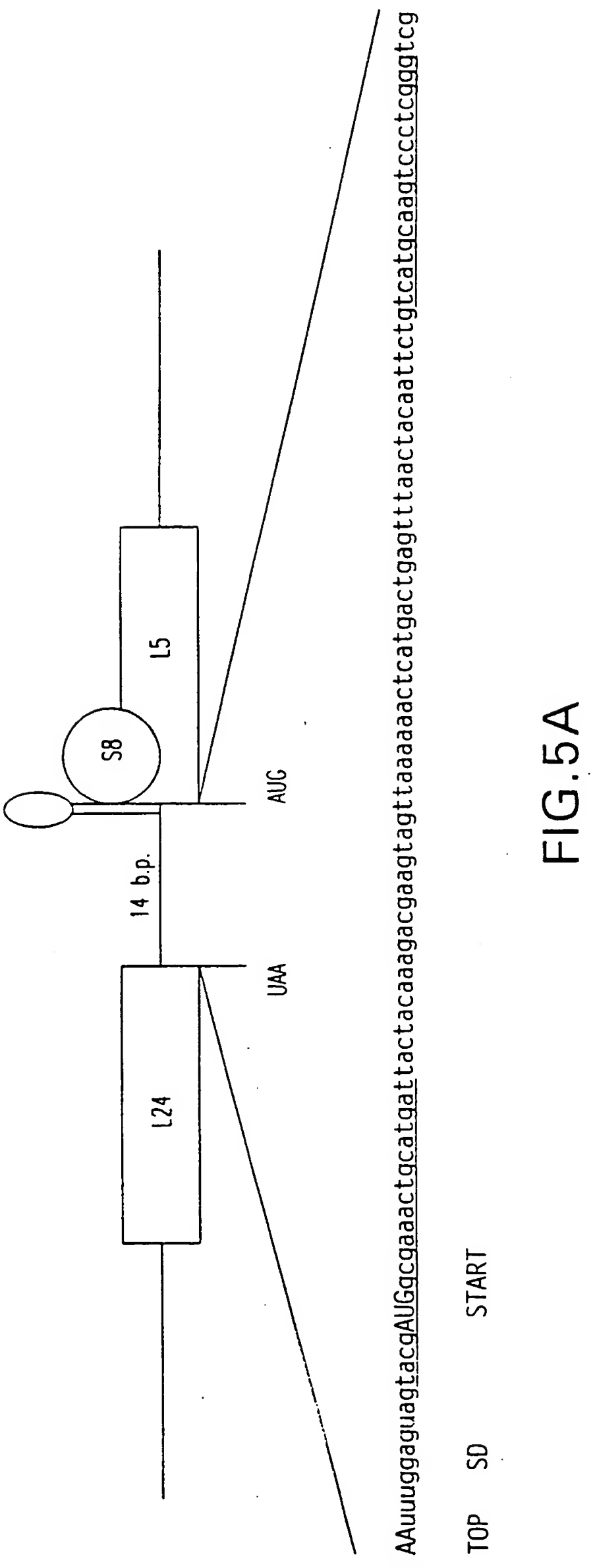


FIG.5A

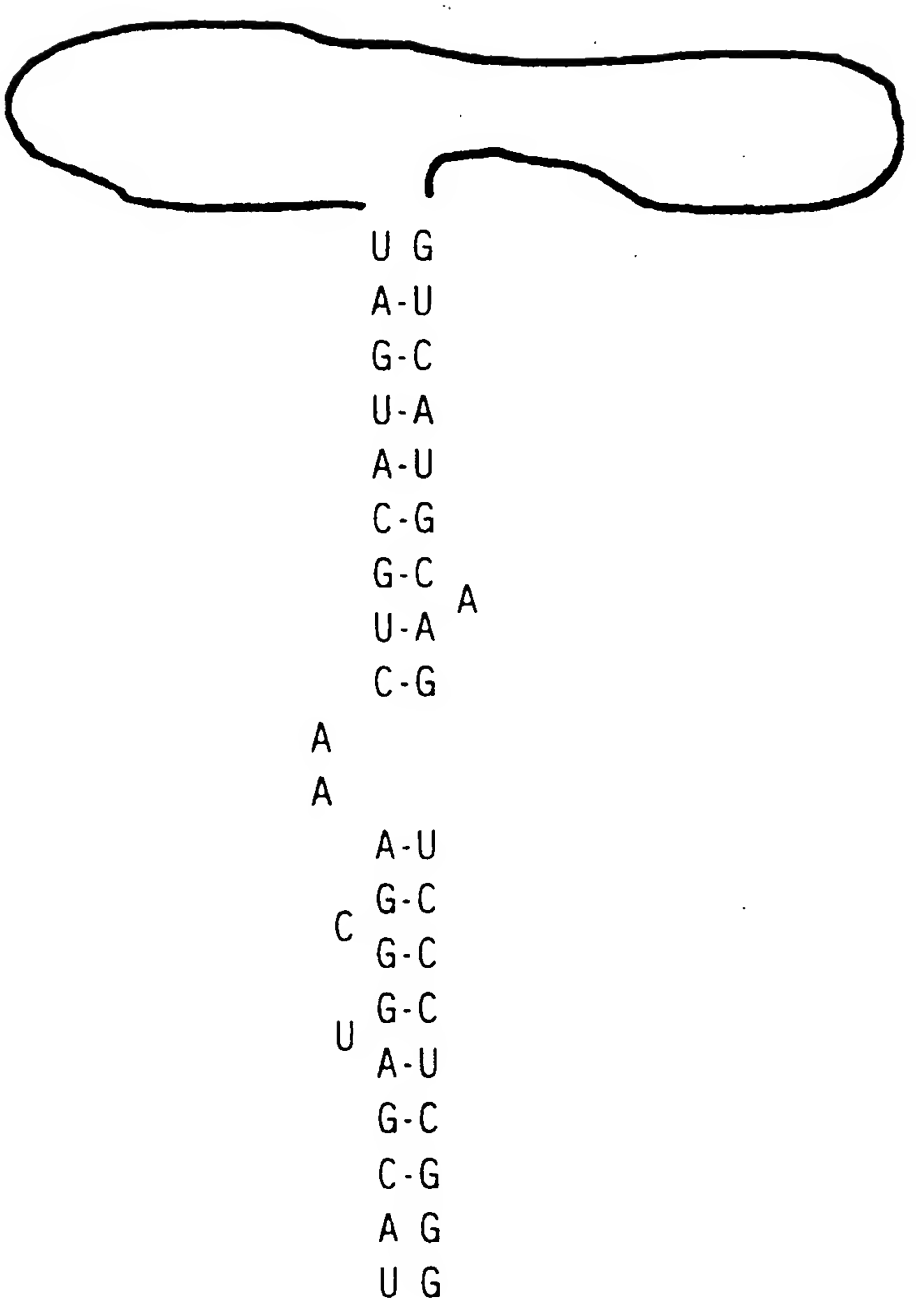


FIG.5B

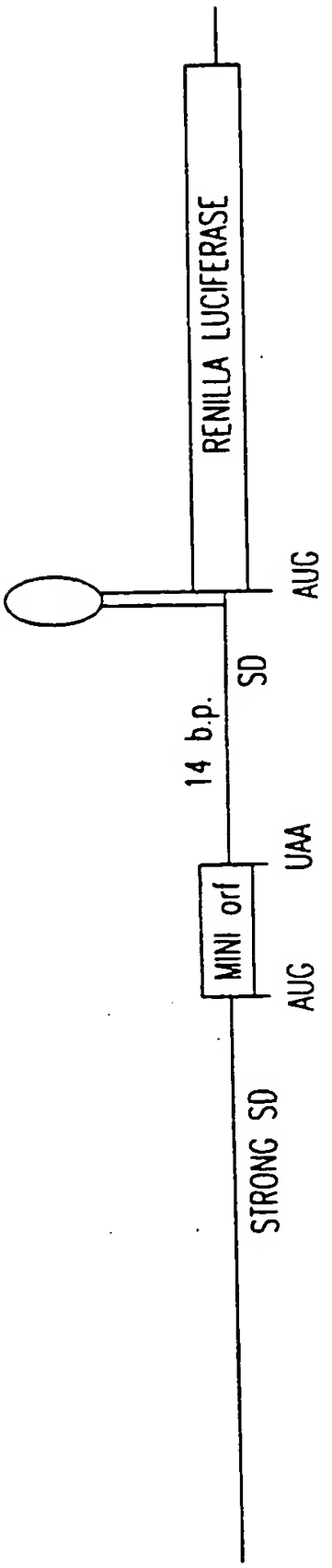


FIG.6A

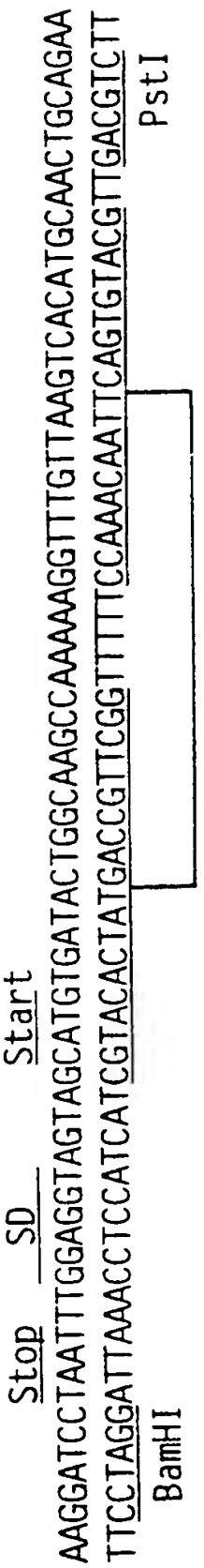


FIG.6B

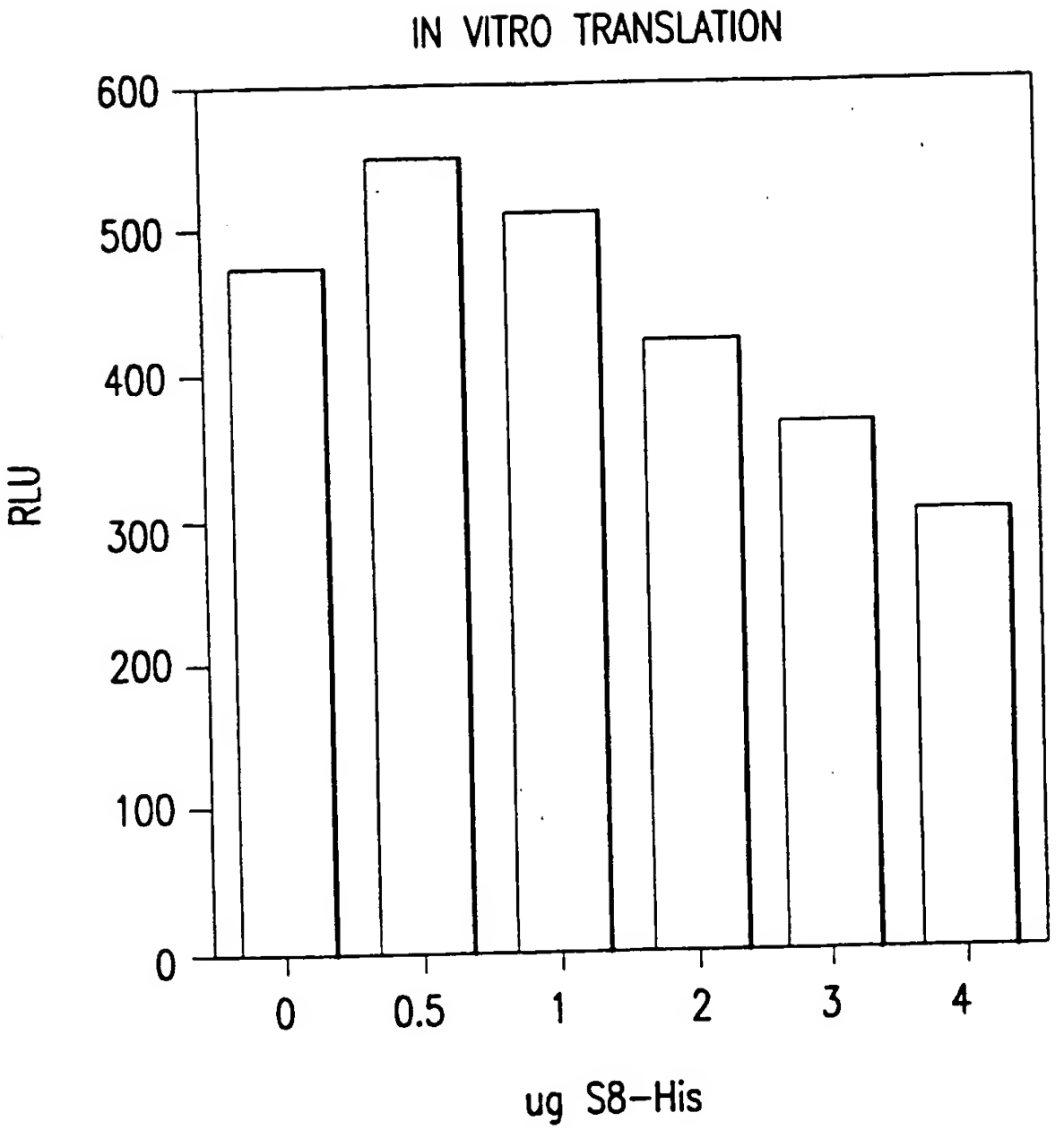


FIG.7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 1/00
US CL : 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.4; 514/29; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GOOD et al. Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. Proceedings of the National Academy of Sciences. March 1998, Vol. 95, pages 2073-2076, see entire document.	1, 3, 4, 7 --- 2, 5,6,8-11,16-25
Y	KARGINOV et al. In vivo assembly of plasmid-expressed ribosomal protein S7 of Thermus thermophilus into Ercherchia coli ribosomes and conditions of its overexpression. FEBS Letters. 1995, Vol. 369, pages 158-160, see entire document.	2,4,6,12,13, 15
Y	PHIZICKY et al. Protein-protein interactions: methods for detection and analysis. Microbiological Reviews. March 1995, pages 94-123, especially pages 96-99, see entire document.	12, 13
Y	BOTTGER, E. C. Resistance to drugs targeting protein synthesis in mycobacteria. Trends in Microbiology. October 1994, Vol. 2, No. 10, pages 416-421, see entire document.	22, 24, 26, 27
Y	MOLLERING, ROBERT C. 'Principles of anti-infective therapy' In: Principles and practice of infectious diseases. Edited by G.L. Mandell et al., New York: Churchill Livingstone, 1995, Vol. 1, pages 199-212, especially pages 206-209, see entire document.	26-31
Y	FIX, J. A. Oral controlled release technology for peptides: Status and future prospects. Pharmaceutical Research. 1996, Vol. 13, pages 1760-1764, see entire document.	20-31

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 February 2000 (03.02.2000)	04 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Ulrike Winkler, Ph.D. Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27839

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAKAKURA et al. Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. Pharmaceutical Research. 1996, Vol. 13, No. 6, pages 820-831, see entire document.	20-31

Form PCT/ISA/210 (continuation of second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US99/27839

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/27839

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Group I, claim(s) 1-17, drawn to a method of screening for an antibiotic.

Group II, claim(s) 18-31, drawn to pharmaceutical formulations and method of treatment.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a method of screening for an antibiotic that interferes with protein synthesis. Group II is drawn to a the pharmaceutical formulation and a method treatment. The prior art discloses antibiotics that interfere with protein synthesis (Botteger E.C. Trends in Microbiology 1994 Vol 2 pages 416-421). Therefore groups I and II lack a special technical feature.

Continuation of B. FIELDS SEARCHED Item 3: WEST, MEDLINE, BIOSIS

ribosomal RNA, ribosomal protein, bacteria, antibiotics, combination drug therapy, macrolides, lincosamide, S1, S4, S7 S8, S15, S20, L1, L4, L10, L20, protein protein interaction, immobilized protein